PHYSIOLOGICAL REVIEWS

VOL. 21 APRIL, 1941 No. 2

CHANGING CONCEPTS OF THE CHEMISTRY OF MUSCULAR CONTRACTION

JACOB SACKS

Laboratory of Pharmacology, University of Michigan Medical School, Ann Arbor

There is no chapter in modern physiology which presents a more interesting development than the one dealing with the nature of the chemical reactions which supply the energy for contraction of striated muscle. New discoveries, each calling for new orientations, have come in such rapid succession as to bewilder and confuse the observer. In the eight years since Hill (39) described the "revolution in muscle physiology" which necessitated a complete revaluation of earlier data, there have been so many new findings that the prediction made therein: "in a few years further discoveries will lead to further drastic change" has been amply justified.

Many of the interpretations in this field have been influenced by a conception of the Pasteur reaction that involves a significant departure from Pasteur's statement of the relation between respiration and fermentation. In the authorized translation of Pasteur's *Etudes sur la Bière* (76) the following occurs:

> . . . fermentation is a chemical action . . . that takes place when these cells, ceasing to have the power of freely consuming the materials of their nutrition by respiratory processes . . . continue to live by utilizing oxygenated matters which . . . produce heat by their decomposition. The character of ferment thus presents itself to us, not as peculiar to any particular being or to any particular organ, but as a general property of the living cell. The character is always ready to manifest itself, and, in reality, does manifest itself, as soon as life ceases to perform its functions under the influence of free oxygen, or without a quantity of that gas sufficient for all the acts of nutrition (p. 114–115).

The modern interpretation of this statement is that fermentation is the fundamental metabolic process, and that respiration is merely a means of decreasing the excessive carbohydrate breakdown inherent in such an inefficient mechanism.

The observations of Hill (36) on the heat relations in muscular contraction were interpreted in these terms. Hill found that the heat liberated during the contraction was the same, per unit of tension developed, whether the muscle were in an atmosphere of oxygen or nitrogen, and drew the conclusion that the chemical reactions which supplied the energy for the contraction were non-oxidative in the presence of oxygen as they must be in its absence. In oxygen, the muscle showed a liberation of heat in the period following the completion of the mechanical event. This was interpreted as evidence of an oxidative recovery process. Since the heat liberation during the recovery period was only a fraction of that which would result from the oxidation of all the lactic acid formed during contraction, the major part must be resynthesized to the glycogen from which it was formed.

Meyerhof (62) then found that the extra oxygen consumed during the recovery period was sufficient to oxidize only one-fifth of the lactic acid which disappeared. This finding led to the concept that the steady state was maintained by continuous anaerobic breakdown and oxidative resynthesis of carbohydrate, with the same amount of lactic acid formed in unit time in the presence of oxygen as in its absence. In oxygen, the major portion was resynthesized to glycogen by the energy liberated in the oxidation of the smaller portion. Thus did the "Pasteur reaction" become the "Meyerhof cycle."

Hartree and Hill (32) then showed that the heat relations were in accord with Meyerhof's chemical data, in indicating that four molecules of lactic acid were resynthesized to glycogen for each one oxidized. They were able to draw up a thermal balance sheet which accounted for all the heat liberated in contraction and recovery in terms of formation and neutralization of lactic acid in contraction, and recovery by the Meyerhof cycle. They found no residual heat which could be attributed to other chemical processes.

This rather simple formulation was disturbed when Embden *et al.* (18) found that under anaerobic conditions the formation of lactic acid continued into the period *after* tetanus. Meyerhof and Lohmann (64) regarded this "delayed" lactic acid formation as an artefact due to "overstimulation." Hill (37) was unable to find any heat production following a series of twitches, and therefore concluded that no lactic acid formation or other exothermic process took place after the contraction was over.

The discovery of phosphocreatine in muscle by Fiske and Subbarow (25) and the finding by Eggleton and Eggleton (17) that it underwent

hydrolysis in contraction and resynthesis in oxidative recovery, initiated a series of investigations which culminated in the "revolution" described by Hill. In addition to the hydrolytic reaction, it was shown (16) that there was reaction between glycogen and phosphocreatine to form the hexosemonophosphate which Embden and Zimmerman (19) postulated as an intermediate in the formation of lactic acid. There was also found (72) resynthesis of part of the phosphocreatine in the post-tetanus period under anaerobic conditions.

Fiske and Subbarow (26) demonstrated that phosphocreatine is a much stronger acid than the phosphoric acid it yields on hydrolysis; the pK'_2 values are 4.6 and 7.0, respectively. They suggested that the function of the hydrolytic reaction in contraction was to supply alkali to buffer the lactic acid formed.

Attention was diverted from this important suggestion by the discovery made by Lundsgaard (54) that frog muscles poisoned with iodoacetic acid could contract anaerobically without the formation of lactic acid. After a number of apparently normal contractions, the muscles passed into rigor and contracture. In the period of normal contractions, the principal reaction taking place was the formation of hexosemonophosphate from glycogen and phosphocreatine; there was also some hydrolysis of phosphocreatine. Lundsgaard used these observations, together with those of Embden (18) on post-tetanus formation of lactic acid, and of Nachmansohn (72) on anaerobic resynthesis of phosphocreatine, as the basis for the hypothesis that the normal source of the energy for contraction is the hydrolysis of phosphocreatine, and that the formation of lactic acid *after* contraction brought about recovery by supplying energy for the resynthesis of phosphocreatine. Thus the first stage in recovery became an anaerobic process, and oxidative reactions were pushed further into the background.

In the normal muscle, most of the phosphocreatine which disappears in contraction undergoes hydrolysis; in the poisoned one, the principal reaction is the conversion to hexosemonophosphate. Lundsgaard at first considered that the formation of hexosemonophosphate from inorganic phosphate and glycogen supplied the energy for the resynthesis of some phosphocreatine, but soon found this untenable. The two pathways of phosphocreatine breakdown were then regarded as being equivalent in function. Meyerhof, Lundsgaard, and Blaschko (67) found that the heat of hydrolysis of phosphocreatine was sufficient to meet the energy requirements of contraction; Meyerhof and Schulz (70) found that the formation of lactic acid after tetanus was not an

artefact due to "over-stimulation"; and Hartree and Hill (33) succeeded in demonstrating the existence of post-tetanus anaerobic heat. This series of events was the occasion for Hill (39) to describe the "revolution" in muscle physiology. To quote from this paper: "Thus it is reasonable to regard the *whole* of the lactic acid set free as being concerned with the restoration of phosphagen broken down during activity. . . ." There remained to be answered the question of why the anaerobic resynthesis was incomplete, for Nachmansohn (73) had shown that it was limited to some 30 per cent of the amount hydrolyzed during the tetanus. There were also some discrepancies in the time relations, for the anaerobic resynthesis which did take place was complete in less than a minute, whereas lactic acid formation continued for more than 5 minutes after the end of the tetanus (50) and delayed anaerobic heat continued to be liberated even longer than this (10).

This formulation had scarcely been completed when another modification became necessary. Meyerhof and Lohmann (65) found that at pH 8.0 to 9.0, the addition of adenosine triphosphate to muscle extract containing polysaccharide led to a reaction in which phosphocreatine and adenylic acid were formed. They postulated that this reaction was the immediate source of the energy for the resynthesis of phosphocreatine, and that the formation of lactic acid at a later stage in anaerobic recovery supplied the energy for the resynthesis of adenosine triphosphate. However, Lundsgaard (57) found that there was no breakdown of adenosine triphosphate in the iodoacetate-poisoned muscle giving single twitches, until after the onset of rigor. He also noted that the poisoned muscle showed additional breakdown of phosphocreatine after tetanus, instead of the resynthesis shown by normal muscle.

Lohmann (53) then found that the phosphate interchange was reversible; at pH 7.0, adenylic acid reacted with phosphocreatine to form adenosine triphosphate and creatine. The hypothesis now became that the initial reaction in contraction was the breakdown of adenosine triphosphate, and that the recovery process began with the resynthesis of this substance by phosphocreatine. At a later stage in anaerobic recovery, the phosphocreatine was resynthesized by the energy of formation of lactic acid, and the final recovery was accomplished by oxidative reactions. In the further evolution of the anaerobic contraction theory, phosphocreatine has been relegated to a still less important position, although it is the one phosphorus compound in muscle which is most likely to undergo change in contraction.

In the experiments referred to above, frog muscle or muscle extract was used. The development by Davenport (15) of the technique of freezing the muscle *in situ* with a mixture of powdered solid carbon dioxide and a volatile liquid, made it feasible to study contraction of mammalian muscle with normal circulation. Application of this technique to the study of the changes produced when muscles of rabbits were subjected to tetani of varying durations, showed that in the first few seconds of contraction there was no hydrolysis of phosphocreatine, but large amounts of lactic acid and hexosemonophosphate were formed (85). The phosphate groups for hexosemonophosphate formation were supplied by phosphocreatine. With longer stimulation, phosphocreatine hydrolysis did appear, and the rates of accumulation of lactic acid and hexosemonophosphate fell off rapidly. When the stimulation period was sufficiently prolonged, there were decreases in the amounts of lactic acid and hexosemonophosphate present, and resynthesis of phosphocreatine was noted. It was difficult to reconcile these findings with the Lundsgaard hypothesis, for several reasons. In the initial period of contraction there was lactic acid formation without phosphocreatine hydrolysis, whereas the reverse situation was to be expected, on account of the postulated lag in anaerobic recovery. Also, the association of phosphocreatine hydrolysis with lactic acid formation gave a relation opposite to the requirements of this hypothesis. Finally, the rapid decrease in the rate of accumulation of anaerobic metabolites did not seem compatible with the idea that contraction is anaerobic. An alternative theory was therefore presented (85): that the fundamental chemical reactions by which the energy for muscular contraction is supplied are oxidative, and that anaerobic reactions are used only when the supply of oxygen is inadequate. The principal anaerobic reaction is the formation of lactic acid from glycogen; whenever this mechanism becomes insufficient, the formation of hexosemonophosphate from glycogen and phosphocreatine is used as a supplementary source of energy. Implicit in this is the concept that hexosemonophosphate is not an intermediate in the formation of lactic acid. The primary function of phosphocreatine hydrolysis is that suggested by Fiske and Subbarow (26), to supply base to neutralize the lactic acid formed in contraction under deficient oxygen supply. Insofar as it is used to form hexosemonophosphate, it serves as a source of energy, but this is limited to the supplementary rôle, and to anaerobic conditions.

Experiments were next made with rats, because Krogh (49) had found that the muscles of small animals contain a larger number of

capillaries per unit cross-section area than is the case with larger species. On this basis, it was possible that the oxygen supply might become adequate more rapidly after the onset of contraction than in the larger animal, and thus the cessation of formation of anaerobic metabolites take place much sooner. The results of the experiments (86) were in accord with this assumption. The accumulation of lactic acid and hexosemonophosphate ceased after only 15 seconds of tetanus in the rat muscles, whereas these processes continued into the second minute in the rabbit experiments. Also, the maximum lactic acid concentration found in the rat muscles was only one-third of the maximum found in the rabbits.

There was more phosphocreatine hydrolyzed in the rat muscles than in the rabbit muscles, relative to the lactic acid formation. The total alkali liberation from the reactions of phosphocreatine in the rat muscles was at least enough to buffer all the lactic acid formed. Most of the alkali was furnished by the hydrolysis of phosphocreatine, but the conversion of phosphocreatine P to hexosemonophosphate P also contributed some base, since this reaction forms an acid with a pK'_2 value of 6.1 (44) from one with pK'_2 value of 4.6 (26).

These calculations were made on the basis of pH 5.6 within the muscle fiber, since the studies of Rous (80) with indicator dyes favored this value, and it is also the pH at which the hydrolysis of phosphocreatine yields the maximum amount of buffer. However, Maurer (59) has shown that the type of indicator dye used by Rous penetrates only into those fibers which it injures. Fenn and Maurer (23) have used the carbon dioxide-bicarbonate and chloride relations of muscle and plasma to calculate the pH within the fiber, and arrive at the value of 6.9 for frog muscle. Hastings and Danielson (34) find pH 6.6 to 6.7 for mammalian muscle by the same method. Recalculation of the data on the stimulated rat muscles to this pH range shows that the alkali liberated by the phosphocreatine reactions is from 90 to 95 per cent of that needed to buffer all the lactic acid formed. A similar calculation from the data of Lundsgaard (56) on a short series of single twitches in frog muscle, on the basis of pH 6.9, shows that the alkali liberated was equivalent to the lactic acid formed. In anaerobiosis of frog muscle where the rate of lactic acid formation is accelerated by the presence of caffeine or dinitrophenol, there is similar equivalence between the alkali liberated by phosphocreatine hydrolysis and the lactic acid formed (13).

In seeking an explanation for the difference in the relative amounts

of phosphocreatine hydrolysis in the muscles of the two species, it was noted that the diet of the rats had an excess of acid-forming components, while that of the rabbits contained an excess of base. This could conceivably result in differences in the amounts of alkali present in the tissues in forms other than the salts of phosphocreatine. If Fiske's theory of phosphocreatine function be correct, then keeping the rabbits on an acid diet might prevent the accumulation of this other source of alkali and thereby lead to increased hydrolysis of phosphocreatine in anaerobic contraction. Parallel experiments were therefore carried out on two groups of young rabbits, one of which had been kept on an acid diet, the other on the alkaline one. It was found that in short periods of tetanus the same tension development, lactic acid formation, and hexosemonophosphate formation took place in the muscles of the two groups, but there was much more hydrolysis of phosphocreatine in the muscles of the "acid" rabbits (87). The total alkali liberation from phosphocreatine reactions in these rabbits was equivalent, at pH 6.6 to 6.7, to 75 to 95 per cent of the lactic acid formed, compared to about 40 per cent in the "alkaline" ones. It would appear that phosphocreatine hydrolysis does not take place until the other source of alkali is much reduced.

As a further test of the buffer theory of phosphocreatine function, the relations in the early part of the recovery period were studied. In cats and in rabbits it was found that the alkali removed by resynthesis of phosphocreatine from its hydrolysis products was practically equivalent, at pH 6.6 to 6.7, to the lactic acid lost by diffusion into the blood stream (88). Thus in contraction and in recovery, the relations found in mammalian muscle were those required by the buffer theory of phosphocreatine function, and opposite to those required by the hypothesis that the energy of formation of lactic acid is used to resynthesize phosphocreatine. It must be pointed out in this connection that Lundsgaard (56) found two moles of phosphocreatine *hydrolyzed* in a series of twitches, and two moles *resynthesized* after a tetanus, for each mole of lactic acid formed.

Additional evidence for the buffer theory of phosphocreatine hydrolysis is furnished by the observations of Lipmann and Meyerhof (52) that muscles, normal or iodoacetate-poisoned, exposed to carbon dioxide tensions greater than physiological, absorb carbon dioxide and hydrolyze phosphocreatine. The absorption is proportional to the concentration of carbon dioxide present and to the time of exposure. The effects are most marked in the absence of oxygen.

Analysis of recovery rates in relation to contraction mechanisms. These experiments on the recovery phase in mammalian muscle furnished a means of testing whether the assumption that lactic acid formation furnished the energy for resynthesis of phosphocreatine could account for the rate of energy production in anaerobic contraction. If this were to function as a recovery mechanism, then phosphocreatine resynthesis must take place with sufficient speed to meet the requirements of the physical situation. In 30 seconds of tetanus by a cat or rabbit gastrocnemius, the lactic acid formed amounts to 150 to 180 mgm. per cent, and in the first 60 seconds after such a tetanus, the resynthesis of phosphocreatine P amounts to 15 mgm. per cent (88). From the known heat of formation of lactic acid from glycogen, 269 cals. per gram (7), and heat of hydrolysis of phosphocreatine, 400 cals. per gram of P (67), it can be calculated that the assumption underlying the Lundsgaard hypothesis would require the resynthesis of phosphocreatine to take place at the rate of 100 to 130 mgm. per cent of P per minute, or more than 12 times the rate actually found. It must again be emphasized that this resynthesis in the recovering muscles is associated with the removal of lactic acid rather than its formation.

A similar calculation can be made for the anaerobic resynthesis of phosphocreatine in frog muscles. This proceeds at a rate not in excess of $\frac{1}{2}$ mgm. per cent of P per second (72). Lactic acid formation in anaerobic tetanus amounts to 4 mgm. per cent per second (24). From the heats of reaction it can be calculated that this rate of lactic acid formation is thermally equivalent to the resynthesis of 3 mgm. per cent of phosphocreatine P per second, or 6 times the maximum rate actually found. Thus the hypothesis that lactic acid formation in contraction serves to resynthesize phosphocreatine requires the assumption that recovery take place much more rapidly during contraction than it does afterward, and that the rate of recovery be greatest when the rate of doing work is greatest. But measurements of the heat production (10) show that the liberation of "recovery" heat is not accelerated by more intense activity. In mammalian muscle it has been found that neither formation of lactic acid nor resynthesis of phosphocreatine takes place under anaerobic conditions in the period following a tetanus (82). Application of the Lundsgaard hypothesis to this situation would require the assumptions that recovery is simultaneous with contraction and that the quantity of phosphocreatine that can be resynthesized anaerobically is limited only by the capacity of the muscle to form lactic acid. In the experiments referred to, the postulated

amount resynthesized would be greater than the quantity originally present.

An alternative explanation has been offered for the anaerobic resynthesis of phosphocreatine that has been observed in frog muscle (88), namely, that this is the means utilized by the muscle to remove alkali from the fiber to compensate for the loss of lactic acid by diffusion into the extracellular spaces. It is well known that diffusion of lactic acid from the muscle fiber is not accompanied by diffusion of base. Unless some compensatory removal of alkali took place within the fiber, the pH would rise considerably. The resynthesis of phosphocreatine from its hydrolysis products is the most efficient and the most readily available mechanism for compensating for this loss of acid. On this basis there is no difference between the resynthesis of phosphocreatine in mammalian muscle which accompanies the diffusion of lactic acid into the blood stream after a tetanus (88), and the resynthesis under anaerobic conditions in frog muscle.

Position of oxidative mechanisms in contraction. Within the past few years there have been some papers in which a more direct utilization of oxygen was indicated than that implied by the Lundsgaard theory. The interpretations in most of these have been made in terms of direct oxidative resynthesis of phosphocreatine instead of anaerobic resynthesis by lactic acid formation. This is the case in the studies of Bang (2) on blood lactic acid in exercise, and of Bugnard (6) and v. Euler (20), working in Hill's laboratory on various aspects of the heat relations of muscle. Gemmill (29) has reported a linear relation between work done and oxygen consumed by isolated frog muscles; this relation is to be expected without reference to whether contraction is fundamentally anaerobic or oxidative, since only the over-all relations are concerned. Flock, Ingle and Bollman (28) conclude from their studies on isotonic contraction of rat muscles *in situ* that contraction is fundamentally oxidative and that the appearance of lactic acid is due to an initial phase of oxygen deficiency.

There is inherent in any chemical method of determining the rate of oxygen utilization by contracting muscle a time lag which is usually fairly long. The development of spectrophotometric methods of recording changes in the rate of oxygen uptake following the onset of contraction, depending on differences in the absorption spectra of the oxidized and reduced forms of oxygen carriers, has reduced this time lag. Urban and Peugnet (96) have studied the reduction of cytochrome *c* in frog muscle, Quensel and Kramer (77) have made observations on the rate

of reduction of oxyhemoglobin in its passage through contracting muscle in the anesthetized dog, and Millikan (71) has investigated the desaturation of muscle hemoglobin in contraction of the cat soleus *in situ* by a technique which reduces the time lag to 0.2 second. The significant finding in these investigations is that the onset of contraction is accompanied by a simultaneous increase in the utilization of oxygen. As Millikan has pointed out, this finding can be satisfied either by direct oxidative reactions, or by anaerobic reactions in contraction which are reversed rapidly by oxidative recovery processes. It is necessary, however, that any postulated anaerobic reaction be reversed with sufficient velocity to account for the steady state.

Oxidative recovery rates from anaerobic reactions. The three anaerobic reactions which need to be considered as possible direct sources of the energy for contraction in the presence of oxygen are: the formation of lactic acid from glycogen, the hydrolysis of phosphocreatine, and the hydrolysis of adenosine triphosphate. It becomes necessary to determine whether the oxidative reversals of these reactions take place with the velocity necessary for the maintenance of the steady state.

With respect to lactic acid, it must be noted that in Meyerhof's experiments, a recovery period of about 24 hours was necessary after 20 to 30 minutes of anaerobic activity (62). This indicates that the oxidative resynthesis of glycogen from lactic acid is very slow. Measurements of the rate of removal of lactic acid by frog muscle show that about 20 mgm. per cent per hour can be disposed of if the concentration present is sufficiently high (12). The last condition is necessary because the oxygen consumption and carbohydrate synthesis of resting muscle are raised by the addition of lactate to the suspending medium (93). Lactic acid formation per twitch by the frog sartorius contracting anaerobically amounts to 0.7 mgm. per cent (24). Hill and Kupalov (41) maintained a steady state of 23 twitches per minute with this muscle in oxygen atmosphere. The assumptions of the Meyerhof cycle would require the rate of lactic acid removal under these conditions to be 16 mgm. per *minute* instead of the 20 mgm. per cent per *hour* actually found. In rat muscles, lactic acid formation in the first minute of activity may amount to 250 mgm. per cent, whereas the maximum rate of glycogen formation in recovery was found to be 3 mgm. per cent per minute (27). To explain the steady state which these muscles maintained after the first minute of activity by the Meyerhof cycle concept would require the assumption that the resynthesis of glycogen is some 70 times as rapid during activity as during recovery. However,

it has been found in both rat (27) and rabbit (89) muscles, that the entire amount of lactic acid formed during a period of anaerobic activity is lost to the muscle during the subsequent recovery period without the appearance of a detectable amount of glycogen in its place. In the rabbit experiments, the total carbohydrate loss in the recovery period was appreciably greater than the amount of lactic acid formed in the contraction; in the rat experiments, no synthesis of glycogen could be detected until after the lactic acid content had been reduced to the resting level.

With respect to the oxidative resynthesis of phosphocreatine: the maximum rate found for this reaction in frog muscle is less than $\frac{1}{2}$ mgm. per cent of P per minute (68). To maintain the steady state of the experiments of Hill and Kupalov by anaerobic hydrolysis and oxidative resynthesis of phosphocreatine would require that the resynthesis take place at the rate of 8 mgm. per cent of P per minute, a rate 16 times greater than that actually observed. In iodoacetate-poisoned frog muscle under anaerobic conditions, phosphocreatine P is converted to hexosemonophosphate P at the rate of 0.3 mgm. per cent per twitch (55). The poisoned muscle supplied with lactate and oxygen can maintain a steady state of activity of 10 (60) or even 14 to 16 (31) twitches per minute, with very little disappearance of phosphocreatine (61). The maximum rate at which frog muscle, normal or poisoned, is able to reconvert hexosemonophosphate to phosphocreatine is 8 mgm. per cent of P per hour (12). To maintain the steady state of the experiments with added lactate by anaerobic formation of hexosemonophosphate and oxidative resynthesis to phosphocreatine would require that the resynthesis take place at the rate of 3 to 5 mgm. per cent of P per *minute* instead of the 8 mgm. per cent per *hour* actually found. In mammalian muscle, a steady state of 2 twitches per second is readily maintained (91). For the energy to be supplied by phosphocreatine hydrolysis and oxidative resynthesis would require the recovery process to take place at the rate of about 70 mgm. per cent of P per minute; the rate actually found following such a steady state was only 5 mgm. per cent per minute (81).

With regard to the resynthesis of adenosine triphosphate, the only data available are for mammalian muscle. It has been found (28, 81) that this process is much slower than the resynthesis of phosphocreatine, and that there is no relation between the two processes.

From all these observations it can be seen that the hypothesis that contraction is anaerobic in the presence of oxygen requires the *ad hoc*

assumption that, at the same oxygen tension, the oxidative reactions take place with much greater velocity in the interval between two successive twitches than they do after the last twitch of the series. If the degree of saturation of the enzyme system concerned were the factor controlling the rate of resynthesis, the opposite relation would be anticipated, since the determinations of removal rate were made with much higher concentrations of substrate present than would be the case under steady state conditions.

With regard to the question of what substances undergo oxidation to furnish the energy for contraction, it must be pointed out that conditions in the normal animal are not suited to the determination of possible mechanisms. It cannot as yet be said with any certainty whether lactic acid, glucose, or hexosemonophosphate is the substance which undergoes oxidation. In the iodoacetate-poisoned muscle, lactic acid is oxidized even when only the normal resting concentration is present (55). It is therefore not unreasonable to assume that normal muscle can also oxidize this substance. Glucose is readily oxidized by the resting muscle poisoned with iodoacetate (3), but there have been no reports on whether glucose oxidation will support the contraction of the poisoned muscle. Ketone bodies can apparently be utilized for contraction, in amounts proportional to the oxygen consumption (5). The evidence with respect to neutral fat or fatty acids is not conclusive.

Mechanism of anaerobic contraction. In anaerobic contraction, the observed changes consist of: *a*, formation of lactic acid from glycogen; *b*, decrease in phosphocreatine content; *c*, increases in inorganic phosphate and hexosemonophosphate contents.

There have been two interpretations of these observed changes. One, which has been derived from the study of the enzyme systems present in cell-free muscle extracts, presents the phosphate interchanges and the formation of lactic acid as interrelated aspects of the mechanism for the energy supply in anaerobic contraction. This formulation is an integration of the work of the Cori, Embden, Meyerhof, Needham, Parnas and Warburg schools. The essential reactions are considered to be the following: glycogen reacts with inorganic phosphate to form glucose-1-phosphate, which rearranges to glucose-6-phosphate. This reacts with adenosine triphosphate to form hexosediphosphate and adenylic acid. The adenosine triphosphate is resynthesized by reaction of the adenylic acid with phosphocreatine, while the hexosediphosphate is split into two molecules of triosephosphate. Both glyceric aldehyde phosphate and dihydroxyacetone phosphate are formed. From this

stage on, there are two pathways. The one, which has been found in both muscle and yeast extracts, involves the oxido-reduction of triose-phosphate to phosphoglyceric and glycerophosphoric acids; the former then rearranges to phosphopyruvic acid which transfers its phosphate group to the adenylic acid which has arisen from the formation of additional triosephosphate. The resulting pyruvic acid reacts with the triosephosphate to form lactic acid and phosphoglyceric acids, the latter then entering the reaction cycle again. The creatine which appeared at an earlier stage is rephosphorylated, either directly by adenosine triphosphate or by phosphopyruvic acid.

The alternative pathway, which to date has been described only for yeast extract—attempts to find it in living yeast were not successful—involves the *non-enzymatic* addition of phosphate to the aldehyde group of glyceric aldehyde phosphate, the oxidation of this to the 1:3 diphosphoglyceric acid by diphosphopyridine nucleotide (cozymase), loss of the phosphate group on position 1, and rearrangement to phosphopyruvic acid. A schematic representation of these reaction cycles and the other reactions which have been found in cell-free extracts, is given by Burk (8).

The other interpretation of the anaerobic phase (81, 85) considers that the observed phosphate changes, the hydrolysis of phosphocreatine and the formation of hexosemonophosphate from phosphocreatine and glycogen, are independent of the formation of lactic acid. The primary reaction which yields energy for contraction under anaerobic conditions is the formation of lactic acid from glycogen by a pathway which does not involve transfer of phosphate groups from either phosphocreatine or adenosine triphosphate. Whenever this mechanism is inadequate, the formation of hexosemonophosphate from glycogen and phosphocreatine is used as a substitute or supplementary reaction. The function of phosphocreatine hydrolysis is that of supplying alkali to neutralize the lactic acid formed.

Analysis of the extract formulation shows that it does not account satisfactorily for the observed changes in phosphate distribution which accompany the formation of lactic acid in muscle. If completed and balanced equations for the reactions are written, the net change is equivalent to the conversion of one hexose unit and one molecule of inorganic phosphate to lactic acid and triosephosphate, with no net change in phosphocreatine, adenosine triphosphate, or hexosemonophosphate. But it is only in extracts in which lactic acid formation does *not* take place, i.e., when an enzyme poison such as iodoacetate or

fluoride is present, that there is a decrease in inorganic phosphate and a formation of triosephosphate. In extracts in which glycolysis does take place, there is always an increase of inorganic phosphate and a loss of phosphocreatine, and in anaerobic contraction there is usually an increase in the hexosemonophosphate content at the expense of phosphocreatine P. If hexosemonophosphate is only an intermediate in the formation of lactic acid, it is difficult to see why it should accumulate most rapidly when the rate of formation of lactic acid is greatest, yet this is the situation found in anaerobic contraction, both in frog (24) and mammalian (91) muscle.

The data obtained on the iodoacetate-poisoned muscle contracting anaerobically are also at variance with the requirements of the extract formulation and the known effects of iodoacetate in extracts. The reactions which this poison have been found to inhibit in extracts are the dehydrogenation of triosephosphate to phosphoglyceric acid either directly (30) or through intermediate formation of diphosphoglyceric aldehyde (1), and the direct resynthesis of adenosine triphosphate from adenylic acid and phosphocreatine (45). The conversion of hexosediphosphate to triosephosphate is not affected, with respect to either the rate of reaction or the equilibrium point reached (66). On this basis, the poisoned muscle contracting anaerobically should show formation of hexosemonophosphate from glycogen and inorganic phosphate, followed by conversion of the monophosphate to the diphosphate by reaction with adenosine triphosphate, and then conversion of part of the hexosediphosphate to triosephosphate. The actual order of events, however, is: reaction of glycogen with phosphocreatine to form hexosemonophosphate, and conversion of the monophosphate to the diphosphate by reaction with either inorganic phosphate or adenosine triphosphate. The third reaction, conversion of part of the hexosediphosphate to triosephosphate, does not take place as long as the cell membrane is intact (83).

These data on normal and iodoacetate-poisoned muscle show that the formation of hexosemonophosphate is supplementary to, or substitute for, the formation of lactic acid, rather than an intermediate stage in its formation. Additional evidence that the two reactions are independent of each other is furnished by the experiments of Kerly and Ronzoni (46) on the effects of varying the pH of the suspending fluid on the resting anaerobiosis of frog muscle. In a medium of pH 6.0, hexosemonophosphate was formed, but no lactic acid, as long as any phosphocreatine remained. When this was exhausted, lactic acid for-

mation took place, but the *accumulated hexosemonophosphate did not disappear*. In a medium of pH 9.0, lactic acid was formed, but no hexosemonophosphate. It was also found (79) that when muscles which had been kept at pH 6.0 until hexosemonophosphate had accumulated and all remaining phosphocreatine hydrolyzed, were transferred to a medium of pH 9.0, the ensuing formation of lactic acid did not lead to loss of hexosemonophosphate.

Additional evidence in point is furnished by the effects of caffeine (13), epinephrine (35), and dinitrophenol (78) on resting anaerobiosis of frog muscle. All of these lead to increased lactic acid formation, and the epinephrine also causes hexosemonophosphate formation. Caffeine and epinephrine together give an additive effect, but with dinitrophenol and epinephrine there results the same lactic acid production that would be caused by dinitrophenol alone, together with markedly augmented formation of hexosemonophosphate (14).

It should be pointed out that the formation of hexosemonophosphate is a means for conserving the glycogen supply of the muscle, for this substance can readily be re-converted to glycogen within the muscle, either under steady state conditions (91) or in recovery (89). Lactic acid, however, is lost from the muscle by diffusion into the blood stream (28, 89). This glycogen conserving mechanism is most in evidence in both frog (24) and mammalian (91) muscle when the intensity of anaerobic work is the greatest.

The observation that a certain mechanism is present in muscle extracts by which lactic acid can be formed, and the finding that relations in the intact muscle are at variance with this mechanism, is not without analogy. One need only recall that tissue slices oxidize the natural *l*-enantiomorphs of the amino acids, while tissue brei oxidizes only the unnatural *d*-forms. The destruction of the cell membrane has resulted in the loss of a property manifested by the intact cell and the appearance of one not shown by the intact cell.

There is no doubt that muscle extracts *can* form lactic acid by mechanisms involving P transport by phosphocreatine and adenosine triphosphate, but this falls short of being proof that the intact muscle *does* form lactic acid by this mechanism. The development of radioactive isotopes has made it possible to trace these interchanges with certainty. Meyerhof (63, 69) and Parnas (75) have shown that the enzymes in muscle extract do not differentiate between radioactive phosphorus and the ordinary stable form. Application of the same technique to the intact muscle of the cat *in situ* (84) has shown definitely that neither

phosphocreatine nor adenosine triphosphate acts as P transporter in the formation of lactic acid in contraction. The only changes of phosphorus compounds found were those which are generally seen: hydrolysis of some phosphocreatine, and conversion of some phosphocreatine P to hexosemonophosphate. These findings also demonstrate that the hexosemonophosphate which is normally present in muscle is not an intermediate in the formation of lactic acid by the intact cell.

The results of the *in vivo* studies with radioactive phosphorus do not give an unequivocal answer to the question of whether all phosphorylating mechanisms are excluded. They do not exclude the possibility of phosphorylation reactions in which phosphate transport takes place through other substances than adenosine triphosphate and phosphocreatine. Ohlmeyer (74) has presented evidence that cozymase can act as the sole P transporter in systems containing hexosediphosphate. Such transport would need to take place by way of the reversible conversion of cozymase, diphosphopyridine nucleotide, to coenzyme II, triphosphopyridine nucleotide, described recently by Adler *et al.* (1). This follows because of Meyerhof (63, 69) has shown by means of radioactive phosphorus that cozymase does not undergo any dephosphorylation in glycolyzing extracts.

There are two major difficulties in this mechanism. One is that participation of adenosine triphosphate is still involved, for it takes part in the formation of hexosediphosphate. The other is that no triosephosphate appears in iodoacetate-poisoned muscles containing large amounts of hexosediphosphate so long as the cell membrane is intact (83), although Meyerhof and Lohmann (66) state that the enzyme poison is without effect on this conversion in extracts. This finding indicates that the mechanism of iodoacetate inhibition of glycolysis is different in the intact cell from that in extracts, and also that different reactions are involved in the formation of lactic acid in the two systems.

The Pasteur reaction and the Meyerhof cycle. The concept formulated by Meyerhof (62) of the mechanism by which oxidative processes prevent the accumulation of anaerobic metabolites is based on the experimental finding that the *extra* oxygen consumption of a recovering muscle is sufficient to oxidize only a part of the lactate which disappears. It is postulated that lactic acid formation takes place to the same extent in respiration as in anaerobiosis, and that respiration supplies the energy for the resynthesis to glycogen of the major part of the lactic acid formed. If the R.Q. of the muscle is 1.0, it would seem more reasonable to relate the lactic acid disappearance to the entire oxygen consumption rather

than to that fraction in excess of the oxygen consumption of a resting muscle. Burk (7) has shown that on this basis the oxidative quotient (lactic acid disappearing divided by lactic acid oxidized) is reduced to 2 instead of Meyerhof's value of 5. From the heat relations involved (7) it can be shown that the value of 10 would be required to account for the steady state, on the basis of 100 per cent efficiency for the resynthesis reaction. An efficiency less than this would mean that lactic acid or equivalent anaerobic metabolites, such as the hydrolysis products of phosphocreatine or adenosine triphosphate, must accumulate in continued activity, or even at rest. The oxidative quotient of 5 would thus mean 50 per cent efficiency for the resynthesis reaction, and consequently that half as much lactic acid should appear in the presence of oxygen as in its absence. This condition manifestly does not hold.

On the other hand, Shorr (93) has obtained high oxidative quotients, in some cases far above the theoretical maximum, with R.Q.'s approaching the level of fat oxidation. Such values are without significance in terms of the Meyerhof cycle concept, since the connection between carbohydrate oxidation and carbohydrate synthesis is absent. It has also been shown (3) that skeletal, cardiac, and smooth muscle poisoned with the minimal effective concentration of iodoacetic acid, maintain an R.Q. of 1.0 in glucose medium, and that the glucose disappearance is equivalent to the oxygen consumption. These theoretical and experimental considerations make it unnecessary to postulate either the anaerobic phase of the Meyerhof cycle or the common intermediate in the anaerobic and oxidative pathways of carbohydrate catabolism. The evidence at hand shows that the synthesis phase of the cycle is potentially present, but it does not have the significance assigned to it by Meyerhof, since it takes place only in isolated tissue systems, but not in muscle with normal circulation.

The Meyerhof cycle concept has led to speculation on the mechanism by which glycolysis, or any fermentation, for that matter, is inhibited by respiration. It is evident that some factor in cell organization imposes limitations on both the respiratory and glycolytic activity of cells. Muscle brei shows much greater lactic acid formation than intact muscle does in the absence of nitrogen, and also shows a much higher oxygen consumption than intact resting muscle. Lipmann (51) has presented evidence for a reversible oxidative inactivation of the glycolytic enzyme system. The validity of this attractive hypothesis is, of course, independent of the Meyerhof cycle concept. Either as it stands,

or with suitable extensions, it may be capable of explaining why certain enzyme poisons (CO, ethyl isocyanide, various dyes) can raise the level of aerobic glycolysis to the anaerobic level without decreasing respiration. The preliminary note by Stern *et al.* (95) on the existence of a "Pasteur agent" may offer the possibility of being this extension of the Lipmann theory. This must await further clarification and the publication of the experimental details. To be valid, it should be capable of explaining why muscle, at rest (48) or during the steady state of activity (91), shows a glycolysis the magnitude of which is related to the oxygen consumption. This continuous glycolysis amounts to about 10 per cent of that which would be found at the same level of activity under anaerobic conditions.

Careful analysis of Pasteur's (76) statement on the relation between respiration and fermentation shows that it does not contain, either explicitly or implicitly, the idea that continuous anaerobic breakdown of carbohydrate is taking place in the presence of oxygen. Pasteur took the position that the living cell utilizes oxygen insofar as it is available, and that fermentation reactions take place only when the supply of oxygen is inadequate to support a fully aerobic metabolism. Yeast, the subject of Pasteur's investigations, clings very tenaciously to the respiratory pathway of metabolism. For example, Warburg and Kubowitz (97) found that at 10°C., the oxygen tension must be lowered to 0.2 mm. Hg to lower the respiration of yeast by 50 per cent. If the view be adopted that respiration is the fundamental metabolic activity, and that anaerobic reactions are resorted to when, and only when, the supply of oxygen is inadequate to meet the metabolic requirements of the cell, much of the mystery surrounding the term "Pasteur reaction" vanishes. An emergency anaerobic mechanism for muscular contraction is obviously necessary, for any appreciable muscular effort involves an initial period of deficient oxygen supply (47). Until the circulation becomes adequate, anaerobic reactions must be called upon to a certain extent, but this does not mean that they are used to the exclusion of direct oxidative ones when sufficient oxygen is available.

Thermodynamics of muscular contraction.[1] It must be pointed out that Hill's (36) finding that the relation between heat liberated and tension developed in contraction is independent of the presence of oxygen, is one which is to be anticipated on the basis of the second law of thermodynamics. In the contracting muscle, as in the steel spring and

[1] The writer is indebted to Dr. H. L. Johnston, professor of physical chemistry, Ohio State University, for this analysis of the thermodynamics of muscle.

the rubber band, the relation between tension developed and heat liberated depends only on the force law involved, i.e., the mathematical statement which expresses the relation between the change in length and the force necessary to produce it. It is immaterial from the thermodynamic viewpoint whether the muscle is actively contracting or being passively stretched, and it is also immaterial whether the source of the energy for contraction is some physical process or chemical reactions. Different sets of chemical reactions which may be the source of the energy for contraction under different conditions can modify the ratio of heat to tension only if they produce changes in the form of the force law or in the numerical values of the constants therein.

It can therefore be seen that if different relations between heat and tension were to be found under aerobic conditions than under anaerobic, this would be a demonstration that different sets of chemical reactions were taking place under the two conditions. On the other hand, if the same heat-tension relations are found, it serves to demonstrate merely that the different chemical processes do not give rise to any change in the force law of the muscle. Only the heat liberated during the mechanical event enters into the matter; the heat liberated after contraction and relaxation are over is a function of the living cell and not of the thermodynamic system. The experimental data show that in every case which has been investigated, the ratio between heat and tension is unchanged. These cases include: normal muscle in oxygen and nitrogen (36), iodoacetate-poisoned muscle in oxygen and nitrogen (21), and curarized muscle in nitrogen (21). The chemical reactions taking place in these muscles are all different except in the cases of the normal and iodoacetate-poisoned muscles in oxygen. It can therefore be stated that the constancy of the relation between heat and tension cannot be used to prove that there is a single reaction, or set of reactions, supplying the energy for contraction under all possible conditions. The data obtained are useful for determining the force law of muscle, but beyond this one cannot logically proceed.

Post-contraction phenomena. There remain to be explained the heat liberation shown by muscle in the period after the completion of contraction and relaxation, and the other phenomena of the post-contraction period. Hill interpreted this post-contraction heat as demonstration of the oxidative recovery from anaerobic contraction. It must be pointed out, however, that smooth and cardiac muscle do not show this phenomenon, and that striated muscle shows it in the absence of oxygen. Analysis of the steady state of activity of striated muscle shows dis-

crepancies between the requirements of Hill's theory and the observed phenomena. If the steady state were to be maintained by anaerobic contraction and oxidative recovery, then the amount of recovery accomplished between two consecutive contractions must be equivalent to that after a single isolated contraction. Otherwise there would be an accumulation of anaerobic metabolites and the steady state would not be present. Now the duration of the liberation of the recovery heat after a single twitch of frog muscle at room temperature is about 40 seconds (38); unless repeated stimulation results in an acceleration of the recovery process, then an activity level greater than one twitch per 40 seconds should lead to the accumulation of anaerobic metabolites. Cut Hill's (38) measurements show that the total time required for the evolution of the recovery heat after n twitches is practically n times as great as after a single twitch, and that the total heat liberation is also n times as great as after a single twitch. This shows conclusively that repeated stimulation does not lead to any significant acceleration of the "recovery" process. Yet Hill and Kupalov (41) were able to maintain a steady state of 23 twitches per minute in the frog sartorius in oxygen, instead of the $1\frac{1}{2}$ per minute theoretically possible. At 0°C., the time for complete evolution of the recovery heat after a single twitch is 30 to 40 minutes (40) and the oxygen consumption shows the same time course (43). On this basis, the maximum steady state activity which should be possible at this temperature would be only two twitches per *hour* instead of the two per *minute* that Hill found to be feasible.

Another observation used by Hill as evidence of oxidative recovery from anaerobic contraction is the extra heat liberated when oxygen is admitted to a muscle which has previously given a series of twitches in nitrogen (38). But this increase in metabolism can be brought about by many processes which do not involve any antecedent contraction. It is observed when lactate is added to the solution bathing the muscle (93), and it appears when the high lactic acid content is produced by resting anaerobiosis. It is seen also after exposure to an atmosphere of carbon dioxide (79). Under these conditions there is hydrolysis of phosphocreatine and formation of hexosemonophosphate. The excess metabolic activity on admission of oxygen to such a muscle is evident as long as phosphocreatine is being resynthesized from its hydrolysis products (79). This effect of the hydrolysis products of phosphocreatine in producing an augmented oxygen consumption can also be obtained with muscle brei (4).

The magnitude of the post-stimulation heat is subject to wide fluctua-

tions under different conditions. It is greater in frog muscle after tetanus than after twitches (9); in mammalian muscle it is much greater at room temperature (23°) than at 37° (11), and it is much greater in mammalian muscle than in frog muscle at the same temperature (11). When the resting metabolism has been elevated by caffeine, the magnitude of the post-stimulation excess of metabolism is much reduced (92). These observations make it difficult to accept the proposition that the post-stimulation heat represents the oxidative recovery from an anaerobic contraction process, for they necessitate the assumptions that the efficiency of recovery is subject to very wide fluctuations with temperature and type of activity, and that it is quite different in frog muscle than in mammalian. It is well known (22) that nerve shows a similar post-stimulation excess of metabolism, amounting to perhaps 20 times the metabolism during the stimulation period. If this did represent a recovery process in nerve, then the efficiency would reach the very low figure of 5 per cent.

Post-stimulation heat and chemical changes, moreover, are not limited to contraction in the presence of oxygen. They occur, to lesser extent, under anaerobic conditions (33). There would seem to be an essential similarity between the post-stimulation excess of oxygen consumption, the post-stimulation formation of lactic acid by frog muscle contracting in nitrogen (18), and the post-stimulation breakdown of phosphocreatine shown by the iodoacetate-poisoned frog muscle contracting in nitrogen (57). In all these cases, there is a continuation into the post-contraction period of the metabolic reactions that took place during the contraction.

There has been offered an alternative interpretation of these phenomena of the post-contraction period, namely, that the tissue which has been stimulated does not return instantaneously to the resting metabolic level (88). The magnitude of this post-stimulation excess of metabolism is related to the duration and intensity of the activity. This explanation is equally valid in interpreting the enormous post-stimulation heat production shown by nerve. The effect is merely of a different order of magnitude than that in muscle.

On this basis, the "oxygen debt" in exercise is merely the manifestation in the entire organism of a phenomenon which is shown by the isolated muscle or nerve. Hill (42) applied the term oxygen debt to the excess oxygen consumption above the resting metabolic level which is found after exercise, and which is related in magnitude and duration to the rate and duration of the exercise. This excess oxygen consump-

tion can be separated into a phase immediately following the cessation of work, which shows rapid decrement, and one which shows slow decrement and long duration. Both phases are present independently of whether there is a rise in the level of lactic acid in the blood. Hill *et al.* (42) considered that the rapid phase represents the resynthesis of lactic acid to glycogen within the muscles in which it was formed, and the slow phase represented the same process taking place in the other tissues into which the lactic acid had diffused. It is known, however, that the limited resynthesis of lactic acid to glycogen which does take place in muscles is a very slow process. Margaria *et al.* (58) assign to the slow phase the function of lactic acid removal, and regard the rapid phase as the oxidative resynthesis to its precursor of some anaerobic metabolite other than lactic acid, since the rapid phase is present even when no increase in blood lactic acid is found. However, no anaerobic reaction has been found in muscle which has the properties of rapid oxidative reversal called for by this hypothesis (81). Also, it has been shown (90) that the lactic acid content of the blood after a short period of steady state activity, such as that used by Margaria *et al.*, is very much lower than that of the muscles themselves. The other phase of the "oxygen debt payment" far outlasts in time that which is required to reduce the lactic acid content of the blood and muscles to the resting level. In view of all these difficulties encountered in relating the post-exercise increase in oxygen consumption to the oxidative removal of anaerobic metabolites, it may be more reasonable to regard it as essentially the same phenomenon that an isolated muscle shows after contraction in oxygen, as a gradual return to the resting metabolic level.

Although there have been many advances in our understanding of the chemistry of muscular contraction during the past decade, many problems still remain to be solved. The mechanism of lactic acid formation within the muscle fiber still remains to be discovered, and the function of adenosine triphosphate awaits elucidation. The whole field of the oxygen carriers involved, and the intermediate stages in the oxidation of the various substrates is just now beginning to be explored. The cytochrome systems are undoubtedly of greatest importance; in fact, Stannard (94) finds that azide and hydroxylamine, specific poisons for this system, do not affect the resting oxygen consumption, but abolish the increase due to stimulation or to caffeine. The participation of the flavine systems is indicated by the spectrophotometric observations of Urban and Peugnet (96).

Finally, and most elusive of all, there remains the question of the

mechanism by which the muscle transforms the chemical energy derived from oxidative or anaerobic reactions, into the mechanical work which is its function in the body.

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RECENT DEVELOPMENTS IN HISTOCHEMISTRY*

I. GERSH

Department of Anatomy, The Johns Hopkins University

Histochemistry deals with the chemistry of tissues. It differs from conventional biochemistry by referring chemical substances to cells and to the pericellular spaces, which together comprise a tissue. Thus, in the analysis of skeletal muscle for inorganic ions, a histochemical problem is the determination of the quantities of each ion present at a particular moment and even in particular places in the muscle fiber, in the tissue fluid space around it and in the connective tissue cells. Again, in studies on nucleic acids, a histochemical problem is the determination in a variety of cells in different functional (and genetic) states of how much is present in the chromosomes, nucleoli and nucleoplasm of the nucleus and in the cytoplasm. The emphasis throughout is on the morphological localization of chemical substances in recognizable tissue space, in cells, and indeed, in the structures of nucleus and cytoplasm. Thus, histochemistry includes certain aspects of cytochemistry. The aims of histochemistry are 1, to define quantitatively the relations of chemical substances located intracellularly with those in their immediate environment, and 2, to determine quantitatively the relations of intracellular chemical entities to the morphological organization of the cell. It is obvious that to the extent to which these goals are approached, new light will be thrown on the functional association between cells in the same or different tissues and on the rôle played by cellular components in metabolic mechanisms.

It is needless to note that the results achieved by histochemistry up to the present time fall far short of the end in view. There are many reasons for this lack of agreement beteeen aspirations and achievements. 1. The concentration of the constituent sought in tissues is usually close to the limit of detectability by the methods employed, when it does not fall below it. 2. Numerous substances are closely bound to other confusing or interfering substances. 3. In both of the preceding classes, phenomena such as diffusion, solubility, denaturation, and inactivation loom large as disturbing factors. 4. In the attempt to visualize selectively one substance present in an intimate and complex

* Submitted for publication June, 1940.

mixture, there are no guiding criteria against which to test the accuracy of, or determine the limitations of the methods employed. A very useful means of estimating the value of a histochemical method is quite pragmatic: those methods are useful which yield results that are in essence in agreement with, or confirmed by, directly related data obtained by means of simpler, chemically valid or physiological (functional) techniques. 5. Technical difficulties incline histochemical work toward a descriptive, qualitative state. It is noteworthy that in the last decade, some of the most significant advances in histochemistry are quantitative ones (see enzymes, nucleic acid).

In the broadest sense, good histochemical work need not be done only with the microscope. For example, the distribution of inorganic substances in striated muscle cells and in the intercellular tissue fluid is significant tissue chemistry, even though it was resolved for the most part by conventional biochemical analyses of whole muscles. Chemical analysis of tissue fluid aspirated from a skeletal muscle may be considered also as histochemistry. Also useful in determining the nature of the environment of the cells of a tissue are chemical analyses of lymph close to its source, of blood as it enters and leaves a capillary bed, etc. Chemical analyses of the fluid carefully withdrawn at known intervals from the lumen of the renal nephron is also histochemistry comparable with conventional histochemical approaches to the problem. Such chemical studies of the immediate environment of the cells of a tissue are reviewed in familiar physiological literature.

This article deals primarily with the more conventional aspects of the histochemistry of higher animals. The material presented is collected under various headings with numerous cross-references: 1, various aspects of metabolism; 2, organs or tissues, and 3, methods. The literature cited is not exhaustive—it was carefully selected from among the most significant contributions in each subject treated. With few exceptions, the period covered is the last decade. References to earlier literature are very adequately treated in Lison's *Histochimie Animale* and in the smaller sectional reviews mentioned in this paper.

INORGANIC IONS. *Chloride, phosphate + carbonate, potassium, calcium, magnesium.* There are two essential requisites for the detection of diffusible substances in tissues: 1. Tissues should be fixed in such a way as to prevent diffusion and preserve these substances very nearly in the position they occupied during life. 2. The particular substance investigated should be visualized in microscopic sections with no significant displacement. Chemical reactions, if used in this process,

should be specific and sensitive, and should avoid destruction or disruption of cellular or intercellular materials. Calcium, magnesium, and silicon are the only inorganic substances which have been detected by other than chemical means (see p. 245). The first condition has been satisfied by the reintroduction of Altmann's (1890) freezing-drying method (1, 33). Parts of animals are removed rapidly from the body and frozen in liquid air or in fluids cooled to temperatures below −125° C. (63). They are then dried in a high degree vacuum chamber kept at −14°C. to −62°C., depending on the substance being investigated (56, 63, 117). After drying, the blocks of tissue are embedded in paraffin and cut with a microtome. Sections of skeletal muscle and several other organs were prepared in this way and then tested for chloride, phosphate + carbonate, and potassium (39). In skeletal muscle, chloride was found only in the intercellular spaces and phosphate + carbonate was visible in the muscle fibers and between them. Potassium was distributed in the same way, but was present in vastly greater amounts in the muscle cells. These observations thus confirmed in a direct, though qualitative manner the distribution of these ions in skeletal muscle that was arrived at from biochemical data (24, 25, 62). These observations are important because they define the nature of the pericellular milieu in muscle and the permeability of the membrane of the muscle fiber. Studies on the distribution of chloride and phosphate + carbonate in the fundic mucosa are discussed on p. 257. A further study showed that when the formation of colloidal calcium phosphate is induced in the circulating blood, this substance is phagocytosed by macrophages of the liver, spleen and bone marrow, and later released (38). The uptake and release of the ingested particles were correlated with chemical studies on the concentration and state of calcium in the blood and in general with certain aspects of calcium metabolism (40, 96). The methods employed in these investigations have been criticized by Scott and Packer (121) as permitting a significant shift in the ions. Their criticism is based largely on theoretical grounds which were clearly defined by Gersh and Stieglitz (43) and by Lison (90). The reviewer, nevertheless, still regards his previous conclusions as valid, in view of his constant awareness of this possible source of error, and because of the uniformity in the results, the support of the essential conclusions by related data obtained with entirely different biochemical methods, and the absence of any demonstration to the contrary.

McLean and Bloom (95), using the v. Kossa method to demonstrate

bone salts in undecalcified serial sections of rat bones, report that in developing bone a zone of uncalcified matrix (osteoid tissue) is a minor phenomenon that is not constantly present and that the wide osteoid borders, the so-called physiological osteoid described in the literature, probably represent a diet deficient in calcium or phosphate. In the resorption of bone they were unable to demonstrate a removal of bone salts before solution of the matrix. In experiments with activated sterols and very large doses of parathyroid hormone, they found calcium salts, mobilized from bone, distributed through the marrow and up to the endothelium. They have been unable to visualize calcium salts passing from vessels to developing bone or cartilage. They have found no evidence of phagocytosis of bone salts by osteoclasts.

Heavy metals. There have been few significant histochemical studies of the rare or heavy elements occurring normally or introduced into the body for medical purposes. The chief reason for this is the insensitivity of the methods employed to demonstrate their presence, especially in view of the low concentration of the elements sought. New methods, or modifications of old ones, have been described in the last five years for the histological detection of bismuth (16, 18), gold (21, 101), silver (104), palladium (102), arsenic (17), iron (131), copper (103, 105), and lead (125).

Microincineration. Microincineration of thin sections of tissues has been developed by Policard and extended to the study of developing, neoplastic, and normal and pathological adult material (117, 118, 119). It has been found that with appropriate care the morphology of the total ash residue for each organ, tissue, and cell is as characteristic as the appearance of the structures in stained preparations. No significant alterations in distribution have been described in different functional states (119). Such changes are, however, recognizable in embryonic and pathological material. In most cells, the ash deposit closely resembles stainable structures; finer details (as mitochondria, neurofibrillae, etc.) escape representation as such in the final picture. Apart from the identification of silicon by virtue of the double refraction of some of its compounds, no other elements could be localized with certainty in ashed preparations until the recent reports by Scott and Packer (120, 121). These workers were able to identify specifically Ca + Mg in "ashed" preparations of striated muscle by observing on the fluorescent screen of an electron microscope the sites which activate the emission of electrons on being heated to 700° to 800°C. These elements were found to be entirely or almost entirely localized in the muscle cells,

chiefly in the "contraction nodes", while little or none could be detected in the tissue fluid spaces. Movement of the more diffusible tissue elements in the stages preceding incineration was reduced to a minimum by preserving the tissue by freezing and drying. Evaluation of observations of incinerated preparations should be tempered by the knowledge that retraction, rolling and rupture of the section as a whole (or of parts of it) may occur during heating (133). Moreover, Godlewski (54) found by direct observation of sections during incineration that following the violent bubbling of paraffin that occurs at lower temperatures (up to about 150°C.), strong intracellular displacements take place as the temperature rises, with the formation of fissures, holes, masses, flakes, filaments and granules. Thus, according to these observations, the ash picture in a well-incinerated preparation may represent the final result of a whole series of previous rearrangements in the organic (and inorganic) constituents of cells that have taken place during the process of ashing the section. This intrinsic source of error may explain the failure after microincineration to detect physiological shifts in inorganic substances; it may also account for the paucity of ash visible in tissue fluid spaces and its absence from the glomerular space, etc. More direct observations of the events that take place during incineration are necessary for an evaluation of the possible artifacts that may take place in such preparations.

Historadiography. It has been found that certain cells and parts of cells exhibit differences in permeability to soft x-rays which can be recorded on photographic plates. For example, nucleoli absorb such x-rays more strongly than the rest of the nucleus, mucigen granules more than myelin or lipid inclusions, cells of the stratum germinativum of the skin more than striated muscle cells, etc. X-ray absorption is thought to depend on the presence in the region photographed of concentrations of elements with low atomic weight or on the presence of heavy metals. The use of this method confirmed the disposition, previously described by means of color reactions, for rather large deposits of bismuth, arsenic, and gold. The method lacks the specificity and precision so essential for critical histochemical work (19, 84, 85, 86, 132).

Of far more significance is the report of Dershem (22) who irradiated microscopic sections of undecalcified bone with nearly monochromatic x-rays arising as a fluorescence from a thin film of scandium activated by soft x-rays. Calcium will absorb emitted light of this particular wave-length almost specifically. The disposition of calcium is then determined from the clear areas of exposed photographic plates. The chief handicap is that the image can be magnified only slightly.

Histospectroscopy. Few significant histochemical contributions have been made by the adaptation of spectrographic methods for the analysis of biological materials (110, 122). The tissue elements capable of analysis have been so large as to make accurate histological localization impossible, except in the case of certain pathological specimens (98, 108, 109). Another limiting factor is that the identification of elements is not quantitative.

ENZYMES. *Pepsin, peptidase, urease, esterase, lipase, amylase.* Ingenious micro-methods were developed by Linderstrøm-Lang and his colleagues to determine in what cells certain enzymes occur, or, in the case of eggs or large unicellular organisms, in what part of the cell they are present. The technique has made possible also an attempt to localize certain other substances in tissues, such as hydrogen and chloride ions (see p. 256), and vitamin C (see p. 251). In the extensive studies on the gastric mucosa of the pig (only urease was studied in the dog), the preliminary operations were always the same. The whole stomach was emptied of its contents and chilled. Small plugs 2.5 mm. in diameter were bored out, and these were cut parallel to the surface in a cold chamber at a constant thickness (25 μ). Enzyme determinations on one or two sections at definitely spaced distances from the surface made it possible to estimate enzyme content throughout the whole thickness of the mucosa. The tissue surrounding the hole formed by the removal of the plug was embedded in paraffin and sectioned serially. Cell counts were then made to determine the numbers of each cell type present at all levels of the mucous membrane. Curves of cell type population at each level were compared with those showing the enzyme content throughout the depth of the glands. Comparisons of such curves showed that the greatest peptic activity was present where the chief cells were most numerous. This is the only unequivocal evidence that these cells are the source of pepsin. Dipeptidase was also found in these cells. Slight peptic activity was present in the pyloric glands, where, in addition, the strongest dipeptidase concentration occurred. In both fundus and pylorus, urease was associated exclusively with the surface cells or with the connective tissue adjacent to them, or with both elements. In the cardia, possible traces of peptic activity and rather large amounts of dipeptidase were associated with the gland cells. Esterase seemed to be present in all cell types of all regions of the gastric epithelium and also, apparently, in the connective tissue. The highest dipeptidase activity was observed in the duodenum, particularly at the levels occupied by the glands of Brunner (49, 87, 88).

Similar analyses of the adrenal gland showed that high lipolytic

enzymic activity seemed to be associated with low content of cellular lipid inclusions. For example, the greatest esterase content, reduced to values for individual cells, was in the medulla and in the zona glomerulosa. The esterase activity of the cells of the zona fasciculata was lowest, while that in the zona reticularis occupied an intermediate position. Lipase activity was greatest in the cells of the medulla, and lowest in the outer part of the zona fascicularis. The activity in other regions of the cortex was intermediate (51).

In all these experiments the conclusions are clear-cut in spite of technical errors in section thickness, in determination of cell volume, in titration, in comparing quantitative enzyme estimations of one group of cells with cell counts on a different, though adjacent group of cells. What is not clear is the degree to which the activity of the enzymes is affected by the impurities transferred to the substrate. It is not known also to what extent the enzymatic activities actually determined are effective in the cell at any one time, and how they may vary from time to time. While these considerations are of no great importance in the work given above, they enter into any evaluation of the cytochemical work summarized below. Large eggs or unicellular organisms were stratified and separated into parts containing different proportions of the formed elements either by continued centrifugation or by microdissection. Holter (71) found in this way that peptidase seemed to be distributed diffusely (in eggs of Arbacia punctulata, Echinorachnius parva and Choelopterus pergamentaceous) in the ground substance of the cytoplasm and not in association with mitochondria. This same distribution of peptidase and catalase was reported also for the egg cells of Psammechinus miliaris (74). Holter and Linderstrøm-Lang found also that the nuclei of Dendraster excentricus and Amoeba proteus were almost or entirely free of peptidase. Later, Holter and Kopec (73) found that peptidase seemed to be distributed in the ground substance of Amoeba proteus also. In contrast with these results, the partitioning of amylase appeared to be different from that of peptidase in this organism, and seemed to be associated with mitochondria, at least to some extent (72). The last study was admittedly subject to larger quantitative errors. Another indication that at least certain enzymes may not be uniformly distributed in the cytoplasm is supplied by the work of Navez and Harvey (100). These workers found that the red halves of centrifuged Arbacia eggs gave a much stronger cytochrome oxidase reaction that the lighter white portions. This was correlated with the greater oxygen uptake displayed by the heavier half

(124). The time is not yet ripe for any definitive opinion on the association of enzymes with cytoplasmic organoids or the nature of this relationship. Technical refinements and the choice of appropriate cells should contribute more toward the solution of this important and vexing problem than the purely descriptive, non-chemical data collected by cytologists until now.

Cytochrome C − cytochrome oxidase + cytochrome B. Recent contributions have gone a long way toward elucidating the nature of the indophenol oxidase reaction. It is caused by the presence of cytochrome oxidase (a part of the cytochrome C-cytochrome oxidase system) + the autoxidizable cytochrome B (81, 129). It should be emphasized that the indophenol reaction as employed on cells is only roughly quantitative, and that the appearance of the dye in cells may not be identical with the localization during life of the oxidase system (90). The cytochrome oxidase system was shown by Friedenwald and Stiehler (31) to play an essential rôle in the selective permeability of the ciliary body to dyes and in the passage of water across it. The respiratory enzyme system is present in high concentration in the epithelial cells of the ciliary body, but absent from the stroma. The use of oxidation-reduction indicators showed that the difference of potential which exists between these tissues disappears when the enzyme is inactivated. This difference of potential thus caused in part by the exclusive presence of the enzyme in the epithelium, gives rise to an electric current bearing anions (such as acid dyes) from epithelium to stroma, and cations (such as basic dyes) in the reverse direction, thus establishing the selective permeability of the organ. It is claimed also that this ionic current is capable of transferring water in the direction from stroma to epithelium. The electric circuit is completed by the stroma-epithelial barrier, which appears to possess a reversible oxidation-reduction system. The cytochrome oxidase complex was found to play a similar rôle in the secretion of the cerebrospinal fluid by the choroid plexus (128). Powerful confirmatory evidence was reported by Flexner and Stiehler (28) when they described the development of this mechanism in the choroid plexus of the fetal pig. In early stages of development of the gland, before secretion has begun, the indophenol-oxidase reaction is equally weak in epithelium and stroma, the potential is equal in both tissues, and acid, basic, and neutral dyes pass equally well in both directions. In later stages, after the onset of secretion, the adult properties of the choroid plexus are gradually established. Similar, though less extensive, findings were reported later for the development of function in the

metanephros of the fetal pig (26). Here again the non-secretory stages did not show the differential distribution of the cytochrome oxidase system or the differences of potential that appear in later secretory stages between epithelium and connective tissue. These workers present an experimental approach and concept that is capable of being extended to other organs of the body, and other developmental processes (see Rulon, 1935).

A further study of the substance responsible for the large reducing capacity characteristic of the ciliary stroma identified the presence of at least two such reversibly oxidizable substances, one of which appears to be ascorbic acid. Vitamin C was found by the silver method to be richer in the stroma than in the epithelium and to be uniformly distributed in the former. Depletion of the vitamin C content of the ciliary body altered the oxidation-reduction potential of the ciliary tissues, the ionic electric current between epithelium and stroma (as demonstrated by the movement of basic dyes) and perhaps the rate of transfer of water. These properties were more or less restored to normal very shortly after the administration of the vitamin (32).

Methods have been described for the localization in tissues of succinodehydrase (123) and phosphatase (55, 130). The presence of phosphatase was noted particularly in the mucous membrane of the intestine, the proximal convolution of the renal nephron, the seminiferous tubules, ovarian follicles, ossifying cartilage and the endothelium of certain regions. A combination of the use of appropriate material and micro-methods for amylase and protease such as those introduced by Pickford and Dorris (106) should extend greatly our knowledge of the factors involved in the development and secretion of these enzymes in gland cells (23).

Sulfuric acid esters. *Heparin.* In the course of a very extensive study of the significance of the metachromatic staining of certain specific tissue elements with dyes like toluidine blue, Lison (92) gave good reasons for believing that this reaction is specific for complex sulfuric acid esters of large molecular weight, such as chondroitin, mucoitin, etc. This made it possible for the first time to attach at least some chemical significance to the large number of studies on cartilage, and other connective tissue derivatives, and "mucous" gland cells, both of which had been known to stain purple with toluidine blue. Later, Holmgren (67) published additional data which suggest that it may be possible to differentiate sulfuric acid esters of low molecular weight from the larger polyesters. This makes feasible an investigation of cytological phenom-

ena associated with the first appearance of precursors of collagen and cartilage. A reinvestigation of the amorphous ground substance of the connective tissue, which stains metachromatically, should lead to a chemical interpretation of this material. Heparin, a complex sulfuric acid ester, was found also to be precipitated and stained metachromatically by toluidine blue. A series of investigations by Jorpes, Holmgren and Wilander (67, 68, 78, 79, 136) disclosed a close parallel in the heparin content of various organs, in the same and different animals, with the number of mast cells present. They believe the granules of these cells, which stain purple with toluidine blue, contain heparin. The tendency of mast cells to be associated with capillaries, and the loss of their granules in peptone shock (when the heparin content of the blood is increased), is further evidence in support of their argument.

VITAMINS. The fact that vitamin C reduces silver salts in acid medium enabled Giroud and his co-workers to localize this compound in tissues (45, 46). The reaction suffers from several grave defects: 1. It is rather insensitive, being negative when the concentration of ascorbic acid is less than one part in 50,000. 2. Certain inhibiting substances (glutathione, epinephrine, cysteine, etc.) reduce the sensitivity still further or inhibit the reaction, notably in the medulla of the adrenal gland. 3. Since the silver salts are far less permeable than vitamin C, the finer localization is open to question, particularly when the former are injected by way of the blood vessels. For this reason it is difficult to attach great significance to the reported distribution of the vitamin in relation to mitochondria, Golgi apparatus, or even in the ground substance of the cytoplasm (47). For the same reason the speculations of Joyet-Lavergne (80) on the respiratory function of the mitochondria are somewhat premature. 4. The reaction is not quantitative. The last objection is largely overcome by the technique introduced by Glick (48). This method, in addition, is more sensitive and specific, and freer from errors due to interfering substances. Using the principles of Linderstrøm-Lang, Glick and Biskind were able to refer their determinations, in a statistical sense only, to individual cells in each zone. In the hypophysis, the cells of the infundibular process were found to be richest in vitamin C; its concentration in the cells of the pars intermedia was greater than in those of the anterior lobe (52). In the adrenal gland, the cells of the outer fasciculata contained the most ascorbic acid, the amount decreasing in the cells of the inner fasciculata, reticularis and medulla; none was found in the capsule or glomerulosa (50). In the thymus, the concentration of vitamin C per unit of parenchyma re-

mained constant throughout the life of the animal (53). In the anterior lobe of the hypophysis, vitamin C was found in all cell types, though present in higher concentration in the chromophile cells. In the ovary follicular cells were found to contain the vitamin only when they were luteinized. In these cells the amount of ascorbic acid increased and remained high as long as the corpus luteum was functional, decreasing as it involuted. Some vitamin C was detectable also in ovarian interstitial cells. In the testis, the vitamin was found chiefly in the interstitial cells, though traces were visible in spermatozoa and Sertoli cells (46). The pericapillary concentration of vitamin C described by the French workers may well be an artifact referable to the mode of application of the silver salts, by vascular injection. The full significance of the high concentration of ascorbic acid in certain organs and cells is unknown at the present time. For the distribution of this compound in the ciliary body and the rôle which it plays in the functioning of that organ see page 250.

Since vitamin A, nicotinic acid and lactoflavin are fluorescent, attempts were made to localize them by means of this property in vital or supravital preparations of liver, kidney, etc. (59, 60, 61, 111, 112, 113). At the present time the results are too qualitative and undiscriminating to assure any real basis for the accurate identification of these vitamins in cells (see Lison, 90). A colorimetric reaction for lactoflavin has been published by Chevremont and Combaire (20), but has not yet been applied.

Nucleus. *Nucleic acid—ultraviolet absorption methods.* Chemical studies of the nucleus made great strides forward with the development of ultraviolet absorption methods by Caspersson (10 and unpublished). Nucleic acid compounds have a characteristic absorption curve in the ultraviolet end of the spectrum, with a peak in the neighborhood of 2600 Å caused chiefly by the presence of purine and pyrimidine rings. Using monochromatic light, with the highest magnifications, Caspersson was able to obtain the ultraviolet absorption curve of a minute volume of cytoplasm or nucleus in microscopic sections or smears by two methods: either directly with the use of a photoelectric cell, or with quantitative photographic plates. The shape of the curve obtained, as compared with that of pure nucleic acid, identifies this compound, and the height of the curve may be used to estimate quantitatively the amount present in the field measured. Observation of the section while protein is being removed by malonic acid or trypsin (in the presence of La^{+++}) aids in the interpretation of the nature of the relations of

nucleic acid with proteins (13). When a crystalline desoxyribose nuclease is available, the nucleic acid-protein association should become clearer. Caspersson's methods may be used to determine quantitatively other substances also (see organic iodine compounds, p. 255) and in time should find wide application.

Caspersson was able to show that nuclear structures which absorb ultraviolet light in the critical region include not only almost all the basichromatin, but also a great part of the oxychromatin. Thus, these two terms should be considered as having only a morphological significance, and not any chemical connotation. He found that nucleic acid in the nuclei of secreting cells of Helix pomatia diminishes during the performance of work, thus confirming chemically the conclusions derived earlier with the use of stains (10). It was found also that in spermatogenesis, the amount of nucleic acid in the nucleus increases during the prophase, and decreases again in the telophase (10, 11, 12). Since this nuclear synthesis of nucleic acid takes place at a time when the distribution of genes into two daughter chromonemata is supposed to take place, a relationship is indicated between gene reproduction and nucleic acid metabolism (14). Caspersson and Schultz also showed that nucleic acid-like compounds occur in the cytoplasm during ovogenesis.

Having established the fact that the dark-staining bands of the giant salivary gland chromosomes of Drosophila are rich in nucleic acid and the pale bands free of it, Schultz and Caspersson were even able to show quantitatively the effects of a localized disturbance in the chromosome on the nucleic acid values of neighboring bands (116). Later, clear evidence was obtained to show that a certain change in the chromosomes was accompanied by an increase in light-absorbing components in the cytoplasm (116). Finally, these authors showed that the cytoplasm of certain rapidly dividing plant and animal cells (which is basophilic) had a high content of pentose nucleic acid-like components, and that this was reduced in later developmental states in which the basophilia is likewise reduced (15). The use of crystalline ribose nuclease should assist materially in a more extensive analysis of the basophilic material present in the cytoplasm of certain secreting cells like the acinar cells of the pancreas. These contributions represent the first encouragingly successful attempts to correlate quantitatively the "closed" system of gene studies with the chemical basis for such a system, to associate genic behavior with cytoplasmic components, and to relate the interphase nuclear changes with cell activity.

Nucleic acid—staining methods. While a positive Feulgen nucleal

reaction is specific for desoxyribose nucleic acid (90), a weaker negative reaction cannot be interpreted as indicating the absence of this compound (9). Moreover, the positive color reaction is so intense as to make quantitative estimations impossible. Color reactions of nucleoproteins were studied from this point of view by Kelley who offered a mechanism for such staining reactions with basic dyes (82, 83). Extensive work on nucleic acid metabolism in cells awaits the development of some such type of staining reaction, which will make possible unequivocal quantitative estimations. Ultraviolet absorption methods are technically difficult, costly and time-consuming. When staining methods are standardized on appropriate test material with ultraviolet absorption studies, progress unquestionably will be more rapid and general. It should be noted that the staining mechanism proposed by Kelley should apply equally well to the localization of nucleic acid compounds in the cytoplasm.

Iron, zinc. Further efforts have been made to evaluate reports on the presence of iron in the nucleus. Policard (107) believes that iron in the nucleus, as reported in the early studies by Miescher *et al.*, is derived from the remnants of cytoplasm which were carried along with the separated nuclei. Miyake (97) and Yakusizi (137) have recently claimed that zinc is also present in their similarly crudely separated nuclear material.

Enzymes. Peptidase was found to be almost or entirely absent from the nucleus (see p. 248). The cytochrome oxidase system has been reported frequently as not being present in the nucleus, but the results are difficult to interpret (see p. 249).

CYTOPLASM. *Ground substance.* The ground substance of the cytoplasm was shown to contain peptidase and catalase evenly distributed throughout the cell (see p. 248). R. R. Bensley (4) has studied the properties of an extract of liver cells prepared with 10 per cent sodium chloride, which he calls plasmosin. Chemically this is a poorly defined substance or group of substances (probably present in the ground substance of the cytoplasm) which he believes may be the basis for such phenomena as reversible gelation, thread formation, fibrillae and other grades of viscosity in the cytoplasm or in some of its structures. The relation to other fiber-forming chemically pure substances such as myosin or nucleic acid is unknown.

Mitochondria. Following preliminary studies (5) on the behavior of mitochondria in liver cells toward various solvents after the organ had been frozen and dried, came R. R. Bensley's remarkable feat of

separating mitochondria from fresh and frozen-dried livers (3). To what extent the mitochondria are contaminated or infiltrated with other cytoplasmic substances is unknown. Mitochondria were isolated in sufficiently large numbers to be analyzed chemically. They were shown to contain proteins, glycerides, cholesterol and perhaps also lecithin. These substances were believed to be highly and evenly dispersed in each mitochondrium though perhaps more concentrated on the peripheral than in the central parts of each organoid.

ENDOCRINE GLANDS. *Adrenal cortex.* In spite of the great need for histochemical studies of the endocrine glands, few significant contributions have been made in recent years. Bennett (2) localized strongly reducing substances (including ketones) in the outer fascicular, lipoid-rich, "secretory" zone of the adrenal cortex by forming phenylhydrazones after removal of ascorbic acid from sections. He believes that the yellow compounds formed in this way represent the phenylhydrazones of a mixture of acetone and alcohol soluble ketones which include the biologically active corticosterones (corticosterone, desoxycorticosterone and dehydrocorticosterone). It should be recalled that the relationship of these chemical substances to the native cortical hormone(s) is not entirely clear. Accepting the conventional concept of growth and repair in the adrenal cortex, he believes that the cortex peripheral to the outer fascicular region in the cat is presecretory, while the deeper parts are postsecretory. No studies on glands in different functional states have yet been reported.

Thyroid gland. Gersh and Caspersson (42) identified organic iodine compounds (thyroxine + diiodotyrosine) and total protein in the colloid of individual follicles in the thyroid gland (see p. 252). This was achieved by determining that the shape of the ultraviolet absorption curves of colloid in microscopic sections is identical with the curves of proteins containing cyclic amino acids such as tyrosine and tryptophane (with their peak at 2800 Å), thyroxine and diiodotyrosine (with their peak at 3200 Å), and thyroglobulin (with peaks at 2800 Å and 3200 Å). A quantitative comparison of the height of the absorption curve (at 2800 Å and 3200 Å) of colloid in different follicles of the same gland showed that there was a great variability in the protein and organic iodine concentration though it was uniform throughout each follicle at any one moment. A change in the mean chemical constitution of the colloid in different physiological states was demonstrated. The results can best be explained by assuming that intrafollicular colloid is formed and reabsorbed continually in the normal and in certain other

functional states, and that in extreme activity organic iodine compounds are secreted basally without entering the lumen of the follicle. Certain cytoplasmic droplets were tentatively identified as consisting of organic iodine compounds.

Pancreas. In the islets of Langerhans, further attempts were made to correlate the solubility (fixability + stainability?) of cytoplasmic granules in the A and B cells with the presence of lipocaic and insulin (S. H. Bensley (6) and S. H. Bensley and C. A. Woerner (7)). It should be emphasized that solution by reagents is not an adequate criterion for identifying chemical substances as complicated as proteins (see Caspersson (10)) and that the concept of cytoplasmic granules as activity carriers does not rest on too firm a basis (see p. 249). The criteria employed in these studies have more validity when considered as morphological rather than as chemical in nature.

Certain chemical studies on the pancreas which are mentioned rarely in reviews on the subject should be emphasized. E. and G. Hammarsten and Olivecrona (57) reported that whereas the normal dog's pancreas contains appreciable amounts of pentose nucleic acid, only traces are found after duct ligation has resulted in complete or almost complete degeneration of the acinar tissue. This was confirmed later by Jorpes (77) when he found that islets in certain fish contain far less nucleic acid than the zymogenic tissue of the pancreas, though the former are richer in phospholipids. These supplement earlier studies showing that islet tissue contains all or almost all the insulin in the pancreas (75, 93, 94, 135).

The distribution of vitamin C in the endocrine glands has been summarized above (see p. 251).

EXOCRINE GLANDS. *Stomach.* Recent histochemical work on the stomach has been concerned chiefly with the determination of the types of cells which produce or contain enzymes (see p. 247), and with the problem of the site and mode of formation of hydrochloric acid. Preliminary studies of the distribution of chloride and of "acid" by Linderstrøm-Lang, Holter, and Ohlsen (87) are necessarily inadequate since the methods they employed cannot prevent (rather, they encourage) the diffusion of both of these substances between the time the organ is removed and the frozen sections are analyzed. The determination of the hydrogen ion concentration of the water in the cytoplasm of the gland cells, canaliculi and contents of the lumina of the fundic glands has been limited because few indicators penetrate the epithelium in sufficiently high concentration to be useful (99). Neutral red has been

used most extensively (58, 64). According to Lison (89) it is difficult to interpret results observed with this indicator because one does not know how they are affected by certain sources of error such as that due to the presence of proteins and salts, to adsorption, to solution of the indicator in lipids, to the formation of intracellular inclusion vacuoles, and to what has been called the "metachromatic error". It is difficult, then, to accept the conclusion derived from work with neutral red (chiefly) that hydrochloric acid is formed in the outer portion of the lumen or in the foveolus of the gastric glands by hydrolysis of a protein chloride formed, stored, and secreted by the parietal cells, whose cytoplasmic water is claimed to be distinctly alkaline (58, 64). Equally inconclusive results were obtained by Lison (91) and Gersh (41). The latter author prevented post-mortem diffusion of chloride by freezing the gastric mucosa in liquid air and drying it in a vacuum at −60 to −62°C. The subsequent procedure visualized chloride in microscopic sections (see p. 244) in the lumen of the glands, in the tissue fluid spaces, and in the zymogenic granules of the chief cells but not in the parietal cells, even when hydrochloric acid was being actively formed. There is as yet no direct evidence that implicates the parietal cell in the formation of hydrochloric acid or that serves as a basis for deriving the mechanism by which it is formed.

Liver. In an analysis of the diurnal rhythm which occurs in the chemical composition of the liver of certain rodents and hedgehogs a Swedish group of investigators developed a method for visualizing the bile components (including bile pigments + bile salts) in the hepatic lobule (29, 30, 65, 66, 69, 70). Bile components are precipitated by Ba^{++} included in the fixative, the precipitate being stained with acid fuchsin. In a comparison of the fluctuation in glycogen, fat and bile content of the liver these workers found that when the glycogen content of the liver is low and glycogen appears chiefly in the cells close to the central vein, the bile components are visible in the wide bile capillaries throughout the hepatic lobule with large amounts present in the parenchymatous cells as granules. When the glycogen content of the liver is high and glycogen is present in most cells of the hepatic lobule, the bile capillaries are narrow and chiefly those at the periphery contain a smaller amount of bile components; the latter cannot be seen in the gland cells or are visible there only in traces. Indirect evidence suggests that a similar process may take place in man also (76). A method as yet untested has been suggested for the histochemical detection of bilirubin (127).

Kidney. For obvious reasons, the conventional type of histochemical work on the mammalian kidney is not quantitative. Hence such studies as do exist are hardly more than suggestive in contrast with the extensive quantitative analyses of nephric function elaborated by Richards and his co-workers on amphibians (114). Ferrocyanide, uric acid, and hemoglobin were found to be eliminated by the glomerulus in a series of mammals (44, 34, 36). There was no evidence that tubular secretion plays a rôle in the elimination of the first two substances mentioned, though it does enter into the excretion of the last substance. The proximal convoluted tubule was shown to be of prime importance in the secretion of phenol red (35). In addition it was found that ferrocyanide is retained in some non-diffusible form in the cells of the proximal convolution weeks after it has ceased to be detectable in the urine. Except for these stored granules, ferrocyanide was not detected in any cells of the renal nephron. This observation was confirmed and used by Van Slyke, Hiller and Miller (134) in an evaluation of renal clearance methods. Their work forms a link in the chain of evidence which supports the use of inulin as a measure of glomerular filtration (126). A comparison of the relative density of ferrocyanide, uric acid, and hemoglobin in different parts of the lumen of the renal nephron led to the conclusion that water is reabsorbed in the descending limb of the loop of Henle. The use of ferrocyanide as an indicator of glomerular function and of phenol red as an indicator of secretory activity in the proximal convolution made possible an extensive study of the functional regression (in the mesonephros) and development (in the metanephros) of renal nephrons in early mammalian embryos of the cat, rabbit, pig and opossum and in the chick. It was found that both the mesonephros and metanephros in these early forms function simultaneously and continuously; urine is formed at a slower rate than in adults. Renal elimination was found to be a continuous function without any interruption due to degeneration of the mesonephros. The onset of tubular elimination of phenol red and of the reabsorption of water in the loop of Henle is accompanied by structural differentiation of these portions of the nephron. The onset of the glomerular elimination of ferrocyanide is not concomitant with any apparent renal differentiation—rather the inception of glomerular function appears to be correlated with extrarenal vascular phenomena. Structural and functional correlations were extended to an analysis of renal function in human embryos based on stained sections (37). Various aspects of these ontogenetical studies were confirmed by later work of Flexner and Gersh (27), Flexner (26), and of Cameron and Chambers (8).

CONCLUSIONS

The last decade has witnessed the development of methods that have made possible histochemical studies on the elusive diffusible elements which occur normally in cells and in the tissue fluid and on complex organic substances present in cells or in their secretions, such as enzymes and nucleic acids. Most important is the trend toward the introduction of quantitative concepts. It is true that the results at hand are for the most part diffuse, rather superficially covering widely varied fields. The continued exploitation of concepts and methods in common use in the physical sciences should obviate this handicap by making possible quantitative histochemical estimations of substances present in concentrations as minute as they are in tissues.

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INTERMEDIARY METABOLITES AND RESPIRATORY CATALYSIS

K. A. C. ELLIOTT

Institute of the Pennsylvania Hospital, 111 North 49th Street, Philadelphia, Pa.

Our knowledge and understanding of the mechanisms which bring about biological oxidation-reduction reactions have advanced rapidly in recent years. One result of this new knowledge is that the distinction between catalyst and substrate has in some respects become less sharp than previously. We may consider, for instance, the reversible oxidation of lactic acid to pyruvic acid by the system consisting of lactic apo-dehydrogenase, coenzyme I, a flavoprotein and methylene blue. The apo-dehydrogenase causes a transfer of hydrogen atoms between lactate (or pyruvate) and coenzyme I (or dihydro-coenzyme I). Coenzyme I is often called the prosthetic, or active, group of lactic dehydrogenase (i.e., of the holo-dehydrogenase), but it may also be regarded as being, like lactate, a substrate of the apo-dehydrogenase (see Dixon and Zerfas, 43). The protein part of the yellow enzyme causes a transfer of hydrogen between coenzyme and flavin nucleotide, the reduced form of the latter being reversibly oxidizable by methylene blue. The protein part of the yellow enzyme may be regarded as having two immediate substrates, namely, coenzyme I (or dihydro-coenzyme I) and the ribo-flavin-phosphate or flavin-adenine-dinucleotide. Only the fact that the flavin derivative has a high affinity for the specific protein, so that the two combined as a "yellow enzyme" were first isolated, has led us to think of the flavin nucleotide rather than the pyridine nucleotide (coenzyme I or II) as the active group of the "holo" yellow enzyme.

Catalysts for the processes of biological oxido-reductions thus fall into two apparently fundamentally different classes. There are the specific proteins, whose mechanism of action is unknown, and the hydrogen carriers, or electron transporters, like the pyridine and flavin nucleotides, whose rôle is well understood. The second class, the carriers, are, in their relations to the specific proteins, scarcely distinguishable from the metabolite substrates. Nevertheless, the pyridine, flavin, and possibly also the thiamine, derivatives serve physiologically purely as catalysts, mediating the oxidation and reduction of metabolites. But

work of recent years has shown that various substances long known only as intermediate products in the metabolic breakdown and synthesis of carbohydrates, proteins, and fats, also play a rôle as catalysts. In many cases the same oxidation-reduction reaction serves either as a step in the metabolism of the substance in question or as a catalytic mechanism in the metabolism of a different, or even of the same, substance. The whole situation illustrates the dynamic interlocking of processes in the living cell, and emphasizes the danger of oversimplification by too precise classification of processes.

In the following pages a number of processes which are both steps in catabolism and in the catalytic machinery will be discussed, reference being made chiefly to recent work on animal tissues.

One or two remarks on the condition of the material studied should first be made. There are essentially five types of tissue preparation commonly used in the type of work to be discussed, namely, the whole organism, the whole organ, slices, disintegrated whole tissue, and isolated or partially isolated systems. Work on whole animals and organs, the most physiological method, tends to give only over-all information, while work on isolated systems, the most analytical method, has provided most of our knowledge of enzymes, coenzymes and isolated reactions. It is work on the intermediate preparations, slices and "breis", which has provided most of the information here to be discussed. Slices approach the physiological condition of the tissue and make possible analytical and gasometric studies which help to throw light on what a tissue normally does. Disintegration of tissue allows various essential materials to be more readily diluted by the suspending medium or destroyed by enzymes, so that their concentration falls below the optimal and their addition produces striking effects which are often not found with slices. Disintegrated tissue therefore helps us to discover essential substances and reactions and to learn about the inter-relation between reactions. However, there is not always an absolute difference between the different methods and information of all kinds has been obtained with all the methods.

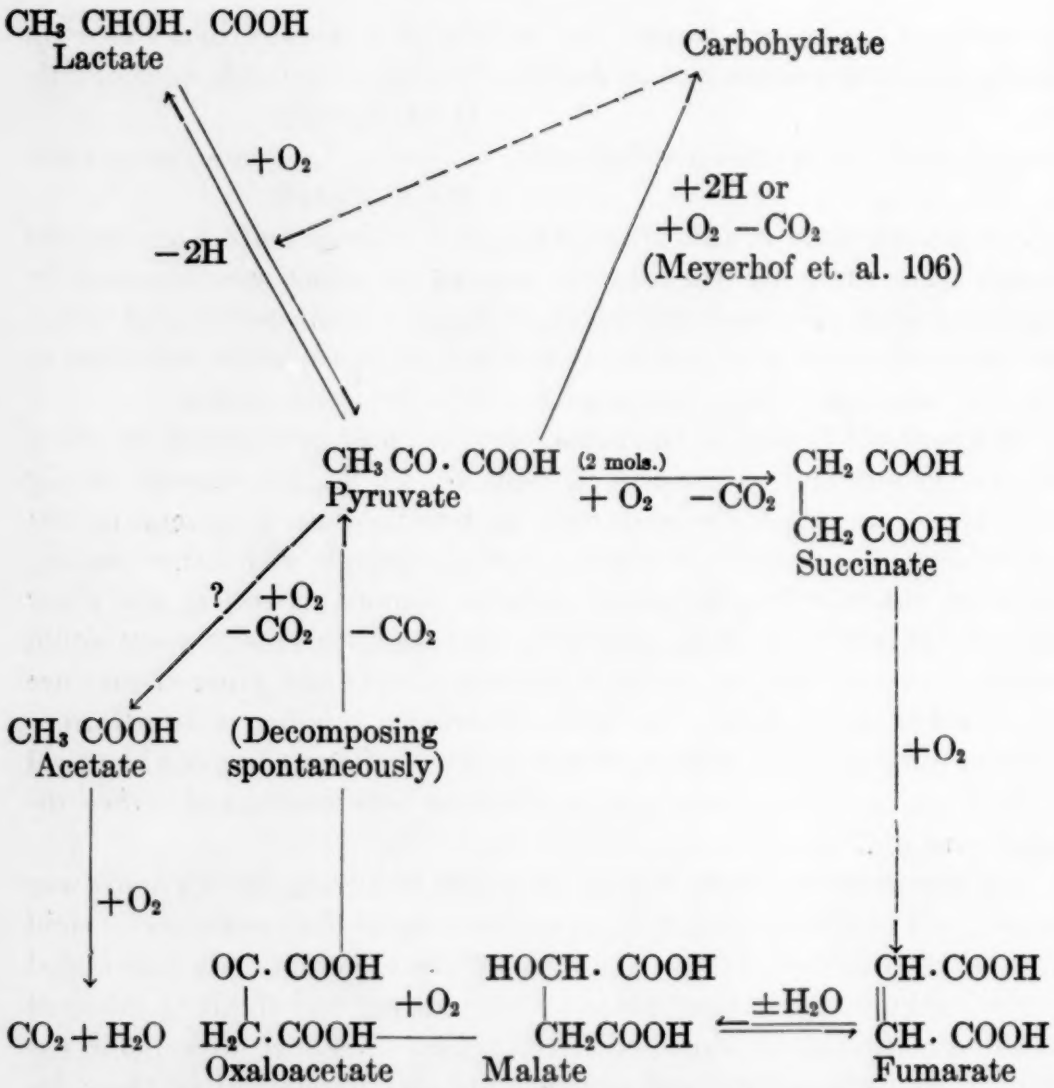
THE FOUR-CARBON DICARBOXYLIC ACIDS. a. *The* C_4 *dicarboxylic acids as intermediary metabolites.* The presence of these acids, succinic, fumaric, malic and oxaloacetic, in animal tissues has long been known. Toenniessen and Brinkman (145; see also Needham, 108) concluded from the results of perfusion experiments that these substances were intermediate products in a cycle of reactions through which lactate was oxidized to CO_2 and water in muscle. According to this scheme, two

molecules of pyruvate, formed by oxidation of lactate, underwent an oxidative condensation and hydrolysis yielding a molecule each of succinate and formate; diketo-adipic acid, $\begin{vmatrix} CH_2CO\cdot COOH \\ CH_2CO\cdot COOH \end{vmatrix}$, was postulated as an intermediate in this transformation. The formate was oxidized away. The succinate was oxidized to fumarate which was converted by fumarase to malate and the latter oxidized to oxaloacetic acid which decomposed yielding CO_2 and one molecule of pyruvate in the place of the two molecules of pyruvate with which the cycle started.

In a series of studies of the metabolism of these compounds by sliced tissues, Elliott and co-workers (**50**, 45, 47, 49, 64, 23) showed clearly that the C_4-dicarboxylic acids can be intermediate products in the breakdown of pyruvate in kidney cortex, though with other tissues, rat liver, brain, testis, and transplantable tumors, ox retina, and chick embryo, this did not seem probable. Satisfactory muscle slices could not be prepared; the reactions in minced muscle and other tissues are discussed in detail later. For kidney cortex a number of transformations of pyruvate were indicated and a cycle similar to, but not identical with, that of Toenniessen and Brinkmann was postulated. (See diagram on p. 270.)

The occurrence of each step in the cycle involving the C_4 acids was proved. The kidney cortex slices caused a rapid disappearance of acid groups when lactate, pyruvate, or any of the C_4 compounds were added to the medium; the oxygen uptake was increased and the R.Q. changed in the direction to be expected if the added substance were being oxidized. With pyruvate and oxaloacetate, rapid removal of these bisulfite-binding substances took place. The formation of considerable amounts of succinate in the presence of added pyruvate, especially when malonate was also added to inhibit further oxidation of succinate, was demonstrated. Good evidence for the oxidation of succinate to fumarate, the equilibrium between fumarate and malate, and the oxidation of malate to oxaloacetate was obtained. Oxaloacetate decomposed spontaneously to pyruvate under the experimental conditions. Formate was not oxidized and it apparently played no part in the cycle.

The cycle did not account for the whole metabolism of pyruvate. It seemed probable that some acetate was formed and added acetate could be oxidized by kidney slices, though not as rapidly as pyruvate itself. More of pyruvate, lactate, and the C_4 compounds was used up than could be accounted for by complete oxidation, and a synthesis of



Transformations of Pyruvate in Kidney Cortex Slices

total carbohydrate was found (Benoy and Elliott, 23) in about the amount necessary to make up the balance sheet of pyruvate disappearance. The fact that succinate, fumarate, and malate, besides pyruvate, also gave carbohydrate synthesis, is added evidence for the cyclic relations of these substances. Of course, lactate, which was readily oxidized to pyruvate, also gave carbohydrate. (The carbohydrate formed appeared not to be glycogen.)

The cycle as given does not show all the steps in pyruvate breakdown. The intermediate steps between pyruvate and succinate were not known, and there are certain problems the solution of which may alter the whole picture. Malate was found to give rise to considerably

more succinate formation than did pyruvate. This could perhaps be accounted for by reduction of fumarate occurring as well as oxidation via pyruvate. The steps after malate require clarification. According to the above scheme, the complete oxidation of added malate depends upon the decarboxylation of oxaloacetate formed, but Elliott *et al.* found no catalyst for this non-oxidative step,[1] and, though added oxaloacetate decomposed rapidly enough to satisfy the scheme, the spontaneous process should be very slow in the low concentration of oxaloacetate which can be produced from malate (see p. 293). Kalckar (73) found that dialyzed kidney extracts took up oxygen rapidly when malate and phosphate were added and phosphopyruvate was formed, fluoride being present to inhibit the breakdown of the latter.

In view of the work on the rôle of citrate, to be discussed later, Greig and Elliott (unpublished results) tried adding citrate and its oxidation product, α-ketoglutarate, to kidney slices. These substances behaved very similarly to the other substances discussed in increasing respiration and being themselves used up. Citrate acid groups were removed considerably more slowly than malate acid groups; α-ketoglutarate was intermediate. This suggests that these substances may be outside the cycle in the same way as lactate is. On the other hand, definite traces of citrate were formed in an aerobic experiment with added pyruvate and somewhat more with oxaloacetate, (—allowing for a slight blank found with oxaloacetate without tissue. Adding pyruvate together with oxaloacetate gave no extra citrate.) Even if formation of citrate were a step in the cycle, a large accumulation of citrate would not be expected since added citrate was found to disappear as citric acid quite rapidly, probably by conversion into *cis*aconitate and *iso*citrate as well as by oxidation. Breusch (35) found a synthesis of citrate from oxaloacetate and pyruvate by minced kidney.

Anaerobic experiments with oxaloacetate gave no clue to its aerobic fate, the results being all accounted for by spontaneous decomposition, slight reduction to malate, slight reduction to lactate of the pyruvate formed on decomposition, and slight extra glycolysis stimulated by pyruvate. No citrate was formed. Aerobically, considerably more oxaloacetate was removed in the presence of tissue than in its absence, indicating an oxidative removal of oxaloacetate by kidney tissue, possibly an oxidative synthesis with some other molecule.

Sliced kidney cortex was the only tissue tried in which pyruvate seemed to be removed by successive transformations through the C_4 dicarboxylic acids. In all the other tissues tested, malate was either not oxidized away or it went too slowly to account for the observed pyruvate oxidation. Nor could formation of succinate from pyruvate in amounts

[1] Breusch (35), however, found a thermostable accelerator of oxaloacetate decarboxylation in minced tissues from all organs tried. The activity was apparently low. (See also Straub, 135.)

comparable with that given by kidney cortex be found, though small amounts were formed in other tissues (see also Weil-Malherbe, 149). Liver slices synthesized carbohydrate from added lactate and pyruvate but not from the other substances. Minced kidney did not behave like sliced kidney cortex.

In view of what follows, the point to be emphasized here is that in kidney cortex slices, pyruvate, succinate, fumarate, malate, oxaloacetate, α-ketoglutarate, and citrate not only increase the rate of respiration but are themselves removed at a considerable rate. Though the oxygen uptake was considerably increased, the amount of acid disappearance could often be accounted for only by assuming that the entire respiration was diverted to oxidizing the added substance and that some of the acid had been synthesized to carbohydrate. There is no doubt that these added substances were not acting purely or mainly as catalysts in kidney cortex slices. With minced tissues there is evidence that the C_4 acids, especially when added in amounts larger than necessary to produce catalytic effects, can be oxidized away at an appreciable rate (see p. 278).

b. *The C_4 dicarboxylic acids as catalysts.*[2] Most of the work on this subject has been done with minced or finely disintegrated[3] tissue, particularly pigeon breast muscle, the latter material being chosen on account of its high respiratory activity and ready availability.

The evidence of cyanide inhibition and of spectroscopic studies has shown that most of the oxygen uptake of the cell takes place through the activity of the "respiratory enzyme" of Warburg, or cytochrome oxidase. This causes the oxidation of cytochrome (the various cytochromes being spoken of collectively) and the cytochrome is reduced again by other constituents of the cell. But succinate with its specific dehydrogenase was, until recently, the only dehydrogenase system known to reduce cytochrome. Succinic dehydrogenase is remarkably active in many tissues and Göszy and Szent-Györgyi (58) suggested that succinate-fumarate with the dehydrogenase acts as a link between other metabolites and the cytochrome-cytochrome oxidase system. (This latter system will be referred to as the WKS, the Warburg-Keilin System.) They suggested that the metabolites activated by their

[2] See Szent-Györgyi's review (142); for another review covering certain recent work in more detail, see Stare and Baumann (130).

[3] The Szent-Györgyi school passed the tissue through a Latapie mill from which the outer grinding disk had been removed so as to obtain the tissue in small pieces rather than completely disintegrated. Krebs and Eggleston (81), however, found that finer mincing made no significant difference.

dehydrogenases reduce fumarate to succinate which is then reoxidized by the WKS.

Szent-Györgyi and his co-workers (58, 7, 6) (see also Stare and Baumann, 129) found that the respiration of muscle suspensions was increased, or rather stabilized at the initial high rate, by the addition of a little fumarate, whereas it fell off otherwise, apparently as a result of loss by diffusion into the medium of fumarate originally present in the tissue. With added fumarate the R.Q. was maintained at the normal value of about 1.0 instead of falling off to a lower value. The added fumarate was not oxidized away. Addition of malonate, which is believed to be a specific[4] competitive inhibitor of succinic dehydrogenase (118), diminished the rate of respiration.

It was found, however, that the malonate inhibition could be overcome by adding fumarate. That is, with added fumarate, respiration continued in spite of the inhibition of succinate oxidation. Since this observation seemed to disprove the succinate-fumarate theory, it was suggested (7, 6) that the C_4 acids derived from fumarate could act as catalysts. Oxaloacetate behaved like fumarate in stabilizing the respiration,[5] reduction of added oxaloacetate to give fumarate and malate occurred very rapidly in the tissue, and oxidation of added fumarate to oxaloacetate could be detected when the oxaloacetate was fixed by hydrazine. From the muscle tissue a strongly active malate[6] dehydrogenase could be obtained. It was therefore suggested that the malate-oxaloacetate oxidation acted catalytically. This system does not react directly with the WKS but a thermolabile "Zwischensubstanz"[7] appeared to be present which acted as intermediate hydrogen transporter between malate[6]-oxaloacetate and the WKS.

An explanation of the effect of malonate + fumarate was later found, and by uniting the two theories (95, 141) all the observations could be fitted together and account taken of the wide distribution and high

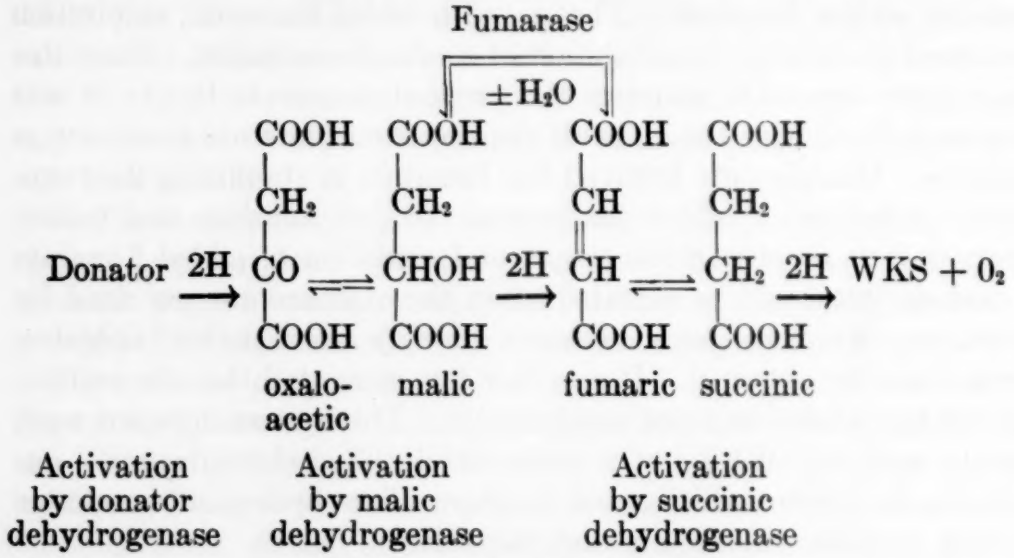
[4] Weil-Malherbe (149) indicated that malonate inhibits other systems, especially in high concentrations (above 0.02 M).

[5] When excess oxaloacetate was added, the O_2 uptake was suppressed for a few minutes until the oxaloacetate was reduced, apparently because oxaloacetate, even in low concentration, inhibits malate oxidation. The observation can be harmonized with the later united theory by Das' (40) observation that oxaloacetate is also a strong inhibitor of succinic dehydrogenase.

[6] At first it was believed that fumarate, not malate, was oxidized to oxaloacetate. See, however, Green (59), Laki (91).

[7] Though Szent-Györgyi (see p. 107 of (6)) later considered the evidence for the existence of the "Zwischensubstanz" to have been incorrect, such a catalyst, diaphorase or coenzyme factor, is now known to exist.

activity of the non-oxidizing enzyme, fumarase, which establishes an equilibrium between fumarate and malate. According to this theory the hydrogen from tissue donators, activated by the relevant enzymes, reduces oxaloacetate to malate under the influence of malic dehydrogenase. The malate, still activated by its dehydrogenase, is then reoxidized to oxaloacetate and the hydrogen is transferred to fumarate, activated by succinic dehydrogenase. Coenzyme I presumably mediates these hydrogen transfers. The succinate formed, still activated by its dehydrogenase, is reoxidized to fumarate by the WKS. Fumarase establishes an equilibrium between fumarate and malate maintaining the right proportions of these substances. The united theory may be represented by the following diagram in which the heavy arrows indicate transfers of hydrogen atoms from one substance to the next.



The Szent-Györgyi Scheme

Measurement of the redox potential of malate-oxaloacetate (Laki, 94) and experiments with isolated systems (Green, 59; Dewan and Green, 42) showed that such a series of oxidations was thermodynamically possible and the transfer of hydrogen from malate to cytochrome through fumarate-succinate was demonstrated (Straub, 136).

The malonate + fumarate effect, mentioned above, was explained by the Szent-Györgyi school as follows (40, 136). Malonate inhibits succinate oxidation because, while it is not itself oxidized, it is more readily adsorbed on the dehydrogenase than is succinate. But fumarate has a greater affinity for the enzyme than has malonate, so it can, to some extent, be adsorbed, activated, and reduced to succinate. The suc-

cinate formed does not in general leave the enzyme surface before it is reoxidized by the WKS. Some succinate must escape, and, being unable to compete with the malonate for return to the enzyme surface, it accumulates as the donator systems of the tissue reduce fresh molecules of fumarate. In this way fumarate is gradually removed from activity and inhibition of respiration sets in early if there is only the original trace of fumarate in the tissue, but no inhibition occurs for a long time if excess fumarate is added. This theory explained the observed accumulation of succinate when fumarate and malonate were present. An earlier explanation of this (6), later abandoned, was that fumarate (malate) was oxidized to oxaloacetate which then suffered an "over-reduction", accepting 4H atoms to give succinate directly by a mechanism unaffected by malonate. According to Krebs (see Citric Acid Cycle), succinate is formed from fumarate in the presence of malonate as a result of an oxidative series of reactions including a synthesis of citrate from oxaloacetate and pyruvate.

Banga (12; see also Greville, 63) fractionated ground muscle suspensions. She obtained a sediment containing the WKS, succinic and malic dehydrogenases, and other enzymes, and a fluid containing the thermostable donator, coenzymes, and an "activator". The "activator" was a protein which was separated by repeated acetone precipitation and appeared to be the dehydrogenase of the donator. The donator could be replaced by hexose mono- or di-phosphate, which is split prior to dehydrogenation into triosephosphate.[8] Washed enzyme suspension + a crude coenzyme preparation[9] + "activator" + hexosephosphate gave a system which respired strongly, especially if an extra trace of fumarate were added, but the oxygen uptake rate varied and usually was not equal to that of normal respiration. But on adding yellow enzyme (Warburg's first yellow enzyme from yeast) the oxygen uptake reached a very high rate. It was concluded that yellow enzyme was necessary to mediate the transfer of H from the malate-oxaloacetate system to the succinate-fumarate system and that traces of yellow enzyme already present in the mixture accounted for the activity when

[8] Banga and Szent-Györgyi (17) have shown that hexosediphosphate is rapidly split to triosephosphate by zymohexase in the sediment. Triosephosphate can replace hexosediphosphate in the system. The "activator" contains triosephosphate dehydrogenase and also a usually less active α-glycerophosphate dehydrogenase.

[9] Presumably the enzyme suspension or the coenzyme preparation contained Mg ions and other possibly necessary factors.

no extra yellow enzyme was added. Yellow enzyme has a redox potential between those of succinate-fumarate and malate oxaloacetate, it is reduced by the malic system, and Laki (93) showed that it is oxidized by fumarate with succinic dehydrogenase.

Straub (136) and Banga (13) have shown that C_4 substances mediate not only oxygen uptake via the WKS but also the reduction of methylene blue and other dyes by unwashed muscle, liver, or kidney tissues. If the succinic dehydrogenase was inhibited by malonate, or if a dye of redox potential too negative to be reduced by succinate but reducible by malate, was used, reduction still occurred but was slower.[10]

Banga found that malonate often had no effect on the rate of methylene blue reduction by well washed tissue + "activator" + coenzyme + hexosediphosphate. However, after dialysis of the tissue, addition of a trace of fumarate accelerated the reduction, and malonate inhibited it. It seeemed that, even after washing, traces of C_4 remained in the tissue (not enough for normal respiration) and these could only be removed by prolonged dialysis, and that the dye reduction, which is slower than the O_2 uptake, is limited not by the C_4 system but by other factors. Szent-Györgyi (141) believed that succinate-fumarate and malate-oxaloacetate are firmly held by the enzymes in a permanently activated condition, that in each case they were dealing not with a dehydrogenase and substrate but with a "transportase" consisting of a protein (the dehydrogenase) and a prosthetic group (C_4 compound). Like the prosthetic group of a yellow enzyme, C_4 can dissociate off. When excess of succinate, for instance, is present, the succinate will continually displace fumarate from the dehydrogenase and rapid oxidation of succinate to fumarate can occur.

The respiration of minced tissues and the effect of fumarate are considerably affected by the composition of the medium. Stare and Baumann (129; see also Greville, 63) found that the addition of heated muscle extracts, presumably containing coenzymes and donators, considerably increased (or maintained) the respiration of minced pigeon muscle and enhanced the effect of fumarate. While the respiration rate per unit weight falls off rapidly on diluting tissue suspensions, Krebs and Eggleston (81) found that addition of muscle extract prevented this fall. With several tissues, Straub and Annau (137) had

[10] With a dye of such negative redox potential the rate of decolorization was *accelerated* by malonate. The fumarate formed by reduction of oxaloacetate evidently accepted H from the reduced dye, i.e., reoxidized it, unless the fumarate-succinate system was inhibited by malonate.

found fumarate effects less marked in Ringer solution than in plain phosphate buffer, but this was probably due to the fact that the Ringer solution contained Ca, whereas the phosphate buffer did not. Ca, in very low concentration, inhibits the respiration and fumarate effect with minced tissues[11] (Greville, 62; Krebs and Eggleston, 81; Elliott and Elliott, 46). Stare and Baumann (129) (see also Krebs and Eggleston 82) found higher respiration rates and stronger fumarate effects in Ringer-phosphate solution (Ca-free) than in plain phosphate buffer. With disintegrated liver Elliott and Elliott (46) found that the respiration was low and added fumarate had no effect unless an adequate concentration of univalent anion (normally chloride) was present in the medium. The effects of NaCl and malate only occurred when the suspension of tissue was fairly concentrated, about 1:10 on wet weight. With more dilute suspensions, other components of the respiratory system were apparenly diluted to less than the optimum concentration. Greville (63; see also 51) found that Mg ions are necessary for the proper respiration of minced pigeon muscle.

Most of the work on this subject has been done with pigeon muscle. The main observations have been confirmed with liver and kidney by Annau (4), Banga (11), Stare (128), and other workers. Malonate inhibits the respiration of minced liver and kidney, and small amounts of added fumarate cause large increases in, and stabilization of, the respiration rate of these tissues. Fumarate is oxidized to oxaloacetate and added oxaloacetate is rapidly reduced to malate. Liver and kidney suspension behaved similarly to pigeon muscle in the reduction of dyes (Straub, 136). Greville (62) showed fumarate and malonate effects with rat diaphragm and sliced brain, and Banga et al. (14) showed that brei or fine dispersion of pigeon brain rapidly reduces oxaloacetate. The respiration of various minced muscles other than pigeon breast,—chicken breast, pig and rabbit heart, rabbit, rat and human skeletal muscle (Stare and Baumann, 129, 130),—have been shown to be increased by the addition of fumarate (or citrate) especially in the presence of boiled muscle juice. Fumarate (or citrate) increased the respiration of Flexner-Jobling sarcoma slices somewhat in the presence of boiled muscle juice; no effect was observed with mince or in the absence of muscle juice. Boyland and Boyland (30) found slight effects of fumarate and malonate with Jensen and Crocker sarcoma. Nitro-

[11] Ca has no effect on the respiration of intact diaphragm (62) or on sliced liver or kidney (81, 46).

phenols increase tissue respiration and Greville indicated that the extra respiration also goes through the fumarate system.

Szent-Györgyi (141) considers it probable that not all metabolites are oxidized through the whole C_4 system. Some may reduce fumarate to succinate directly without the mediation of oxaloacetate-malate. Lactate, for instance, has about the same redox potential as malate and so would not be expected to reduce oxaloacetate.

The experiments of Banga (12; 17) with fractionated tissue materials showed that hexosephosphate (triosephosphate) and, usually less actively, α-glycerophosphate, are oxidized through the system. She also mentioned that alcohol, citrate, and lactate were oxidized by the system and their oxidation inhibited by malonate. Annau showed that the presence of fumarate was necessary for the normal aerobic oxidation of pyruvate by liver suspension (5) and that succinate was necessary for the reduction of methylene blue by pyruvate with washed tissue suspensions (8). Banga, Ochoa, and Peters (14) found that the C_4 substances,[12] together with inorganic phosphate, adenine nucleotide, cocarboxylase, Mg^{++} (or Mn^{++}), and probably cozymase, are necessary for the proper oxidation of pyruvate by dialyzed fine dispersions of pigeon, rabbit, or guinea pig brain or of rabbit kidney cortex (see also 38).

Determinations of R.Q. (Stare and Baumann, 129; Banga, 11; Greville, 63; Elliott and Elliott, 46) and of acid disappearance (Elliott and Elliott) have shown that, with minced tissues, the effects of small additions of fumarate or malate are almost entirely catalytic, though, when larger amounts of these substances are added, they are themselves oxidized away to some extent (see also 134, 70, 9, 67). Usually the R.Q. is about unity, indicating that mostly carbohydrate materials are being oxidized. Greville showed that fumarate is concerned in the oxidation of glycogen by muscle dispersions but that normally not more than 70 per cent of the respiration brought about by fumarate is due to oxidation of carbohydrate. With livers of fasted rats, Elliott and and Elliott found low R.Q. values for the extra respiration caused by adding malate, indicating that added malate was catalyzing the oxidation of non-carbohydrate material, probably fat derivatives. Leloir and Muñoz (96) found that added C_4 dicarboxylic acids (and citrate) increased the rate of butyric acid oxidation by liver suspension. Dewan

[12] Lipmann (97) found that "protein" preparations from *B. delbrückii* required, for the oxidation of pyruvate, cocarboxylase, Mn or Mg or Co, and phosphate, and also flavin-adenine-dinucleotide, but did not require added C_4 substances.

and Green (42), with isolated enzyme systems, showed the oxidation of β-hydroxybutyrate by fumarate. Annau (4, 5) showed by qualitative tests that small amounts of acetone bodies were formed by liver slices or mince without addition, and considerably more when pyruvate was added. The inhibitor, malonate, increased the accumulation of acetone bodies and the addition of fumarate diminished it. Kidney also formed acetoacetate in the presence of pyruvate and malonate. Elliott and Elliott confirmed quantitatively a rapid accumulation of acetoacetate from pyruvate with liver (only in the presence of NaCl), and decreased amounts when a little malate or oxaloacetate was present. Some acetoacetate was also formed from lactate and citrate. Annau concluded that fumarate catalyzed normal oxidation of pyruvate, and in its absence acetoacetate was formed by less normal condensation and oxidation; but Elliott suggested that fumarate catalyzes the oxidation of acetoacetate.

What proportion of the normal respiration of various tissues is carried through the C_4 system is not decided. Most normal tissues contain enough succinic dehydrogenase and WKS to account for the whole normal respiration through the C_4 system (Elliott and Greig, 48; see also discussion in 64). Malic dehydrogenase was present in all tissues tested by Green (59). On the other hand, chick embryo, thymus, spleen, pancreas, and certain rat tumors are deficient in succinic dehydrogenase (45, 48, 64). Breusch (35) found strong oxaloacetate reduction with minced muscle, liver and kidney, some reduction with brain and pancreas, but negligible oxaloacetate (or pyruvate) reduction with spleen, lung, placenta and peripheral nerves. Also certain rat tumors (Banga, 11) and embryo tissue (Blaszo, 26) reduce oxaloacetate or pyruvate scarcely at all.

Quastel *et al.* (119) had shown that *B. coli* could grow anaerobically with glycerol or lactate plus fumarate in the medium, the fumarate serving as H acceptor for the oxidation of the substrates. Krebs (76) produced evidence that, in *B. coli*, fumarate catalyzes the aerobic oxidation of glucose, malate, lactate, acetate, glycerol, glyceraldehyde, butyrate, pyruvate, acetoacetate, *l*(+)-glutamate, and molecular hydrogen. The oxidation of lactate, malate, and of formate could take place independently of fumarate but most of the aerobic oxidation of the other substances seemed to be mediated by fumarate-succinate. Oxaloacetate also appeared to be a hydrogen carrier in *B. coli*.

It is now known that diaphorase, coenzyme factor, makes possible the transfer of hydrogen from donators, activated by coenzyme-determined dehydrogenases, directly to the WKS without the mediation of

the C_4 system. Though most diaphorase preparations contain succinic dehydrogenase, Potter (117) has reported that malonate does not inhibit oxidation of dihydro-coenzyme by a diaphorase preparation (containing cytochrome and cytochrome oxidase) and additions of fumarate did not accelerate diaphorase action. There does not seem, therefore, to be any necessity for the C_4 system. Nevertheless, there is sufficient evidence to show that C_4 dicarboxylic acids do play a catalytic rôle in respiration and the relation between mediation by the Szent-Györgyi system and by diaphorase remains to be determined.

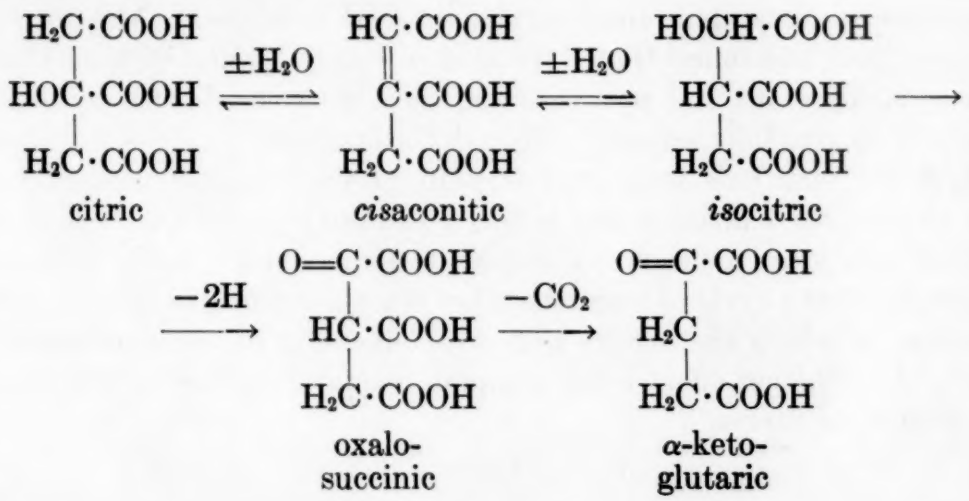
Fischer and co-workers (57) have discovered an enzyme, fumarate hydrase or ethylene hydrase, which brings about the reduction of fumarate to succinate by certain leuco-dyes or by leuco yellow enzyme and possibly by dihydro-coenzymes. Through the yellow enzyme (possibly without it) fumarate hydrase could therefore cause the reduction of fumarate by any of the coenzyme-determined dehydrogenase systems. Fumarate hydrase was shown to be different from succinic dehydrogenase. It apparently belongs to the group of flavoproteins or yellow enzymes. It is present in yeast, bacteria and plants; if it exists in animal tissues it may provide another mechanism for the transfer of hydrogen from donators to the succinate-WKS system.

The different theory discussed below offers an alternative or supplementary explanation of facts discussed above and of certain other observations.

CITRIC AND α-KETOGLUTARIC ACIDS AND THEIR DERIVATIVES. a. *Citric and α-ketoglutaric acids as intermediary metabolites.* Citric acid is widely distributed in common foods and is constantly present in small smounts in tissues and excreta, especially urine. Various authors have shown that when citrate is administered orally in large amounts, only a little is recovered in the urine, so that it is clear that citrate can be metabolized. MacKay *et al.* (102) find that citric acid, like succinic acid, is a glucose former and is as effective as glucose in the relief of insulin hypoglycemia in rats. Citrate can also be synthesized from endogenous materials, since, when alkali is given to animals, more citrate may be excreted than is ingested in food. Orten and Smith (111), who should be consulted for references to earlier literature, (see also Smith and Meyer, 125; Martensson, 103) found that of 21 substances injected into dogs, only succinate, fumarate, malate, malonate, and maleate gave rise to large excretions of citrate. With these substances, and with oxaloacetate, Krebs *et al.* (87) obtained large increases of citrate and α-ketoglutarate in the urine of rabbits. Simola *et al.* (124) found that rats excreted large amounts of citrate and α-ketoglu-

tarate when fed the C_4 dibasic acids, or pyruvate, α-ketoglutarate, β-hydroxybutyrate, acetoacetate, or various other aliphatic acids. With humans, oral administration of large doses of sodium pyruvate gave rise to the excretion of large amounts of α-ketoglutarate; the citrate excretion was raised also, but not more than by the administration of equivalent amounts of sodium bicarbonate. With rats, vitamin B deficiency caused a raised excretion of α-ketoglutarate which was reduced on administration of vitamin B_1 (Simola, 123).

That mammalian and other tissues can oxidize citrate rapidly has been known since the work of Battelli and Stern. Bernheim (24) described an active enzyme preparation from liver, and, using this preparation, Martius (104) showed that the main course of citrate oxidation probably occurs as follows:

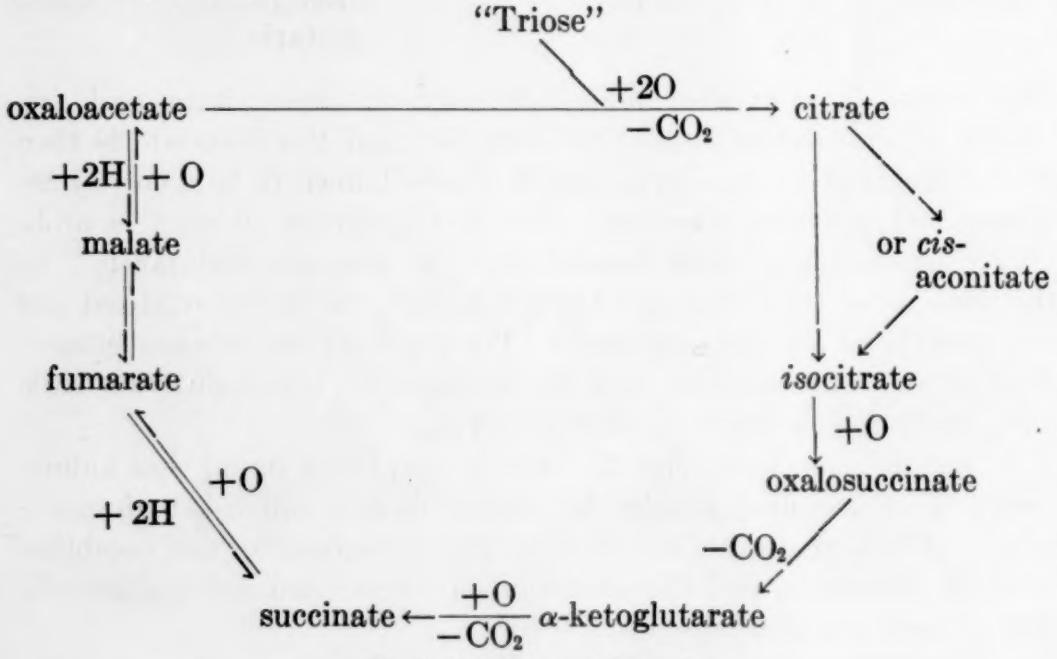


The citrate is converted non-oxidatively to *cis*aconitate and *iso*-citrate by an enzyme similar to fumarase, and the *iso*citrate is then dehydrogenated by an enzyme which is now known to be a coenzyme-determined dehydrogenase (2). The first oxidation product is probably oxalosuccinate which breaks down to give α-ketoglutarate. In the presence of other enzymes the α-ketoglutarate can be oxidized and decarboxylated to give succinate. The equilibrium between citrate, *cis*aconitate, and *iso*citrate, and the oxidation to α-ketoglutarate have been confirmed by other workers (34, 71).

As was mentioned on page 271, Elliott and Greig found that kidney cortex slices are able rapidly to oxidize citrate and α-ketoglutarate away. The formation of citrate from various substrates and combinations of substrates, and the oxidation of citrate and α-ketoglutarate, with minced tissues are discussed below.

b. *The citric acid cycle of Krebs.* Krebs and his co-workers (84, 81)

found that added citrate or *iso*citrate behaved similarly to the C_4 dicarboxylic acids in catalytically promoting the respiration of pigeon muscle suspensions, especially if glycogen, hexosediphosphate, or α-glycerophosphate were also present. Since citrate promoted respiration catalytically—that is, caused a greater O_2 uptake than its own oxidation could account for,—Krebs and Johnson believed that citrate was oxidized and regenerated. When arsenite or malonate was added, it was found that citrate disappeared. Arsenite inhibits the oxidation of α-keto acids and, in the presence of arsenite, accumulation of α-ketoglutarate in the expected amount was found. With malonate to inhibit succinate oxidation, the expected amount of succinate was found. Finally, on incubating oxaloacetate with minced muscle anaerobically, they found a synthesis of citrate. The two additional C atoms were presumed to have come from carbohydrate derivatives. (Martius and Knoop (105) had found that citric acid is readily formed without tissue when oxaloacetate and pyruvate together are treated with hydrogen peroxide in alkaline medium.) Though the synthesis involves oxidation, anaerobic conditions were necessary to prevent further oxidation of the citrate; the oxidation was believed to take place at the expense of reduction of other molecules of oxaloacetate to malate. Krebs believed, therefore, that a cycle of reactions takes place according to the following diagram in which the arrows indicate conversion of one substance to the next. The net effect is the complete oxidation of the carbohydrate derivative, or "triose".



Krebs' citric acid cycle

According to this scheme, succinate can arise from added oxaloacetate both by oxidation via citrate and by reduction via malate. As was therefore to be expected, they found that, aerobically, added malonate increased the yield of succinate from oxaloacetate by preventing further oxidation, while anaerobically malonate decreased the yield of succinate by interfering with fumarate reduction. All the substances of the citric acid cycle were equivalent in maintaining the respiration of muscle mince.

Krebs, Salvin, and Johnson (87) considered that the theory was supported by the observations mentioned above that injection of the salts of the C_4 dicarboxylic acids gave rise to increased excretion of citric and α-ketoglutaric acids. The fact that malonate had similar effects was explained as due to inhibition of citrate breakdown. Malonate is not itself metabolized but inhibits succinate oxidation, and as was expected, when malonate was administered, succinate was found in large amounts in the urine.

Some of Krebs' experimental results have been confirmed by other workers, but his results and conclusions have also been subjected to considerable criticism.

Breusch (34) and others observed the increase of respiration by citrate, *cis*aconitate, *iso*citrate, and α-ketoglutarate, and Baumann and Stare (22) found that the C_4 dicarboxylic acids, citrate, α-ketoglutarate, and glutamate (which is presumably converted to α-ketoglutarate by transamination), all caused total increases in the oxygen uptake of pigeon muscle greater than could be accounted for by simple oxidation of the added substances. Also with other muscle tissues, and with minced or sliced kidney (only in the presence of glucose) or liver, citrate (and fumarate) caused more sustained respiration, though the increase in oxygen uptake was not always sufficient to prove catalysis (130). But citrate was usually not as effective as fumarate; and Stare and Baumann (130) noted that higher concentrations of citrate (0.01–0.02 M) often inhibited respiration somewhat. Tables of Krebs and and Eggleston (81), and Long *et al.* (101), also show this effect. Thomas (144) and Stadie *et al.* (127) did not obtain sufficiently increased oxygen uptakes with pigeon muscle and citrate to prove a catalytic effect of the latter. Thomas found that the effect of citrate was small and was apparent only after a latent period whereas the effects of the C_4 acids were immediate. Krebs (78) points out that, when Thomas found no accelerating effect of citrate, his figures also did not show the well-confirmed catalytic effect of the C_4 compounds. Krebs also believes

that many of the criticisms of Thomas and Breusch (see below) are not serious since their figures for normal oxygen uptake were lower than his, indicating that the experiments were made with tissue which had lost much of its metabolic activity. Krebs and Eggleston (81) had found effects of citrate and α-ketoglutarate as immediately apparent as those of the C_4 compounds; Elliott and Elliott found the same with liver. However, the effect of citrate does seem to be variable.[13]

Breusch (34) was unable to confirm the synthesis of citrate from oxaloacetate and tissue metabolites in pigeon muscle. He indicated that citrate was only found when abnormally high concentrations of oxaloacetate were used and that it arose artificially from a condensation product which is formed when oxaloacetate is neutralized in strong solution. Later he (35) found that minced kidney (aerobically or anaerobically) causes a condensation of oxaloacetate with pyruvate, glycerate, or dihydroxyacetone to give citrate. Minced liver, brain, and lung showed slight synthesis under anaerobic conditions, but he still found no citrate formation by muscle (cat or pigeon). No citrate was formed by kidney mince when the other C_4 substances, or α-ketoglutarate, glutamate, or various other substances were added instead of oxaloacetate. The formation of citrate from oxaloacetate by kidney was found to compete unfavorably with the reduction to malate. Citrate formation appeared to occur only above a certain threshold of oxaloacetate concentration and Breusch suggested that citrate formation was chiefly an elimination process for the disposal of surplus C_4 dicarboxylic acids, these not being excretable in the urine but convertible via oxaloacetate into citrate which can be excreted. Smith and Orten (126), as a result of citrate determinations in blood and tissues in normal and nephrectomized animals, had concluded that citrate production occurred chiefly in the kidney or was dependent on the presence of the kidney.

Breusch's claims that, with muscle, *a*, citrate arose from an impurity of oxaloacetate, and *b*, was found only with unphysiologically high concentrations of oxaloacetate, and *c*, in low amount, were answered by Krebs (78, 82) with the observations that in aerobic experiments, in the presence of excess pyruvate which suppresses the oxidation of other

[13] Krebs and Eggleston (81) stated that catalytic effects with the C_4 acids and citrate were not always found at pH 7.4 but regularly at pH 6.8. This statement seems to be contradicted in the same paper (table VII). Stadie *et al.* worked at pH 6.8 but did not obtain high enough increases in oxygen uptake to prove catalysis. In his later papers (82, 78) Krebs apparently worked at pH 7.4.

substances (see below), *a*, fumarate also gave citrate, and *b*, oxaloacetate would only be formed gradually by oxidation of fumarate, and that *c*, yields of 15 per cent of the maximum citrate were obtained. Higher yields of citrate were not to be expected since citrate is rapidly further oxidized and citrate removal is accelerated by oxaloacetate;—citrate is oxidized by oxaloacetate in the Szent-Györgyi manner (Krebs 79, see below). Simola and co-workers (65) found appreciable production of citrate by minced tissues from various organs with various substrates. With minced ox heart and brain, under aerobic conditions, citrate was found to be produced especially strongly in the presence of pyruvate plus fumarate, malate, or oxaloacetate. Hallman and Simola (66) consider that, at least in heart muscle, which can decompose citrate rapidly, pyruvate is decomposed by the citric acid cycle.

Breusch (34) and Thomas (144) found that citrate added to muscle tissue disappeared rapidly, even in the absence of arsenite or malonate, whereas regeneration through the cycle should maintain its concentration. But later Breusch (35) criticized the cycle theory with the observations that, while liver and kidney mince could break down citrate, muscle scarcely had any such activity. Krebs (78) pointed out that earlier workers had shown an active citrate oxidizing system in muscle and he calculated from Breusch's earlier figures that the citrate oxidation rate was more than sufficient to account for the total respiration rate of the tissue. Krebs believed that the oxidation of citrate can be demonstrated when the physiological balance is upset, as by adding excess citrate or an inhibitor of the cycle, otherwise regeneration via the cycle can prevent the observation of citrate oxidation.

Breusch (34) considered that, according to the cycle, more malate should be produced from added citrate than from oxaloacetate, but the reverse was found to occur. Thomas (144) found the formation of malate from added oxaloacetate was as rapid under anaerobic as under aerobic conditions, whereas the presence of oxygen would be required if the malate was to be formed by the citric acid cycle. The yield of malate was not diminished by the presence of malonate; malonate, by inhibiting succinate oxidation should decrease malate formation via the cycle. Krebs (84, 78), however, has always agreed that the reactions among the C_4 acids are readily reversible and, as will be shown below, reduction of oxaloacetate to malate plays an important part in the later more developed scheme of Krebs (79).

Breusch (34) found that addition of citrate was much less effective than fumarate in overcoming the malonate inhibition of respiration.

Baumann and Stare (22, 131) found the same thing with citrate and glutamate, though α-ketoglutarate was as effective as the C_4 substances. The reason for this was not clear, but since citrate did not behave like the other substances in this respect, it apparently could not be an essential part of a catalytic cycle with the C_4 substances.

Thomas (144) found that, in the presence of arsenite and dinitrophenylhydrazone, added malate, fumarate and succinate gave rise to the rapid formation of the pyruvic hydrazone, indicating a rapid formation from the C_4 substances of oxaloacetate, which broke down to pyruvate. But citrate did not give oxaloacetate (i.e., the pyruvic hydrazone) rapidly. Szent-Györgyi believes that arsenite inhibits the reducing donator systems and these results were interpreted to mean that, with the reducing systems inhibited, the C_4 acids but not citric acid are oxidized to oxaloacetate. If Krebs' citrate theory and his belief that arsenite inhibits the oxidation of α-ketoglutaric acid (or any α-keto-acid) were correct, α-ketoglutarate and not oxaloacetate should have accumulated from the added C_4 acids.

With liver suspensions, Elliott and Elliott (46) found that the respiration (in the presence of NaCl in the proper concentration) was approximately equally accelerated by lactate and pyruvate as well as by citrate and the other members of Krebs' cycle. They suggested that added lactate, pyruvate, citrate, and α-ketoglutarate are oxidized, yielding the C_4 substances, and that the latter act as catalysts in the manner suggested by Szent-Györgyi; Breusch (34) has made the same suggestion regarding the effect of citrate with pigeon muscle.

With dialyzed pigeon brain dispersions, in the presence of coenzymes and other additions, Banga, Ochoa, and Peters (14) found that the C_4 dicarboxylic acids were active in promoting pyruvate oxidation but citric and α-ketoglutaric acids were much less active. Citrate itself was not oxidized by the dialyzed dispersions with all the additions, nor had it been found to be oxidized by brain brei (101, 35). Nevertheless, brain does contain the citric (*iso*citric) dehydrogenase system (2, 71), and, as mentioned above, Breusch (35) and Simola (65) have found some citrate synthesis in brain.

The last few points against the citric acid theory have not yet been experimentally answered but suggestions concerning some of them are made in section *d*. Krebs (78) emphasizes the oxidative formation of succinate from oxaloacetate as conclusive evidence for the cycle. In the presence of malonate and low concentrations of oxaloacetate or

fumarate, pigeon muscle produced six to seven times more succinate aerobically than by anaerobic reduction.

Krebs has recently brought forward new evidence supporting and elaborating his theory.

Krebs and Eggleston (82) found that minced pigeon breast muscle in Ringer-phosphate medium, caused the rapid disappearance of added pyruvate, the rate falling off almost completely in the third or fourth hour. Some increase in the rate of oxygen uptake occurred, especially after about an hour. The total oxygen uptake, and the R.Q. found, corresponded approximately to complete oxidation of the pyruvate utilized and indicated that the oxidation of all other substrates had been suppressed. Malonate in high concentration (0.025M) inhibited the aerobic utilization of pyruvate by 92 per cent, the remaining 8 per cent being due to anaerobic processes unaffected by malonate. In low concentration (0.001M) malonate caused only 77 per cent inhibition. The addition of fumarate completely abolished the effect of low malonate but with higher malonate concentration fumarate caused a pyruvate utilization approximately equivalent to the amount of fumarate added. An additional uptake of 2 to 2.5 mols. of O_2 per mol. of fumarate added, occurred in the presence of high malonate (whether pyruvate was present or not, see below); 80 to 100 per cent equivalent of fumarate was recovered as succinate, and about 3 mols. of CO_2 were given off. An anaerobic experiment showed that the malonate effectively prevented any appreciable reduction of fumarate to succinate, so it was concluded that the succinate arose by an oxidative process according to equation (1).

$$\text{Fumarate} + \text{pyruvate} + 2O_2 = \text{succinate} + 3CO_2 + H_2O \quad (1)$$

Deviations from the theoretical values were attributed to some oxidation of fumarate to oxaloacetate, the latter decomposing to pyruvate and CO_2, some oxidation of lactate to pyruvate, and slightly incomplete inhibition of succinate oxidation. It was believed that when the malonate concentration was low, some oxidation of succinate through pyruvate could occur and reaction (1) would be repeated.

Equation (1) was believed to be the net effect of the following series of reactions:

$$\text{fumarate} \xrightarrow{+O} \text{oxaloacetate} \xrightarrow{\text{pyruvate} + O} \text{citrate} + CO_2$$

$$\xrightarrow{+O} \alpha\text{-ketoglutarate} + CO_2 \xrightarrow{+O} \text{succinate} + CO_2$$

Supporting this belief was the observation that approximately the expected amounts of oxygen uptake and succinate formation occurred, in the presence of malonate, with oxaloacetate + pyruvate, with citrate, or with α-ketoglutarate,[14] but not with a number of other possible intermediates. With fumarate and excess of pyruvate, yields of 50 per cent of the maximum possible amount of α-ketoglutarate and 15 per cent of citrate[15] were recovered; presumably the excess pyruvate, being preferentially utilized, suppressed further oxidation of these substances. Even without added pyruvate, some succinate and α-ketoglutarate were formed, the necessary pyruvate being formed from tissue carbohydrate.

The fact, mentioned above, that in the presence of high malonate even without added pyruvate, about 2.5 mols. of extra oxygen were taken up per mol. of fumarate added, and most of the fumarate was recovered as succinate, was taken to indicate that the oxidation of normal substrates behaves as pyruvate oxidation, and that the above reaction series represents the main steps in the bulk of the normal respiration in muscle, in which carbohydrate is known to be the chief substrate.

Very recently Krebs (79) has brought forward evidence which largely harmonizes his theory with that of Szent-Györgyi. In his opinion, the Szent-Györgyi theory of oxaloacetate ⇋ malate serving as a hydrogen carrier, and the citric acid cycle as a series of steps in intermediary metabolism, are complementary processes. In the oxidation of carbohydrate by pigeon muscle, the first oxidative step is the oxidation of "triose" to pyruvic acid. (Presumably diphospho-glyceraldehyde is the substance actually oxidized,—Negelein and Bromel, 110; Warburg and Christian, 148.) As was shown by the Szent-Györgyi school (6,

[14] Krebs' table gives only O_2 uptake with α-ketoglutarate since succinate could not be determined in the presence of α-ketoglutarate and malonate. No data can be found to show directly the rapid formation of succinate from α-ketoglutarate in muscle. But there is ample evidence that citrate (*iso*citrate) is oxidized to α-ketoglutarate, that succinate can be produced from citrate, and that α-ketoglutarate shows effects on respiration similar to those of citrate and succinate. It is, therefore, inferred that citrate is oxidized to succinate via α-ketoglutarate. The formation of succinate in other tissues by dismutation of α-ketoglutarate with itself, or with acetoacetate, or with α-iminoglutarate is mentioned on pages 295 and 299.

[15] Krebs' figures for "citrate" include citrate, *cis*aconitate, and *iso*citrate, these substances being rapidly interconvertible in tissue, without any oxidation involved, and give an equilibrium mixture containing about 80 per cent citrate, 4 per cent *cis*aconitate, and 16 per cent *iso*citrate (Johnson, 71).

see also Parnas and Szankowski, 115), oxaloacetate accepts the hydrogen, yielding malate which can be reoxidized. Then pyruvate + oxaloacetate undergo oxidative synthesis to citric acid, and the hydrogen equivalent of the oxidation is, according to Krebs and Johnson (84), accepted by another molecule of oxaloacetate. The citrate formed is oxidized to α-ketoglutarate, oxaloacetate again accepting the hydrogen. Krebs established this last point by finding that, anaerobically, added citrate alone was not removed, but it was rapidly removed when oxaloacetate was added, and an approximately equivalent amount of α-ketoglutarate was formed; the rate was much greater than was necessary to account for the total respiration through the citric acid cycle. Krebs believed that oxaloacetate might be the H acceptor for the oxidation of α-ketoglutarate to succinate, but he was unable to demonstrate this.[16] Thus oxaloacetate accepts three and possibly four out of six pairs of hydrogen atoms in the oxidation via the cycle of a molecule of triose. Of course all these hydrogen transfers are mediated by the coenzymes[17] under the influence of dehydrogenases. The malate in each case is reoxidized to oxaloacetate, the hydrogen being transferred to the cytochrome system and oxygen through coenzyme I, either via diaphorase or possibly through fumarate $\rightleftharpoons$ succinate.

The scheme in its present form is by no means the whole story of carbohydrate oxidation since details concerning the synthesis of citrate and the rôle of phosphorylations have not yet been worked out. The "triose" which is oxidized is a phosphorylated compound. With kidney extracts, Kalckar (73) observed the formation of phosphopyruvate when malate was being oxidized and Ferdmann and Epstein (55) report formation of phosphopyruvate when lactate is oxidized by cat muscle. Colowick, Welch and Cori (38) have found that a phosphorylation of glucose is linked with the oxidation of succinate. Lipmann (98, 99, see however 113) has suggested that, at least in *B. delbrückii*, the oxidation of pyruvate is actually an oxidation of a pyruvate-phosphate compound to acetylphosphate, the latter being able to transfer phosphate to adenylic acid leaving free acetic acid. Ochoa (112) finds that oxidation of pyruvate by brain dispersions is coupled with phosphorylation of adenylic acid.

c. *Insulin effects.* Krebs and Eggleston (81) found that in the pres-

[16] See footnote 14 on page 288.

[17] Krebs points out that *iso*citric dehydrogenase uses coenzyme II, while only dihydro-coenzyme I reduces oxaloacetate to malate; the mechanism of the reaction between citrate and oxaloacetate is therefore not yet clear.

ence of boiled muscle juice (see p. 276) and substances of the citric acid cycle, the addition of small amounts of insulin caused a further large increase in the respiration of minced pigeon muscle. The effect, which was most marked with citrate, appeared more as a stabilization of the inital high rate over a longer period than as an actual increase in the initial respiration rate.

This effect has been confirmed in principle by Shorr and Barker (122), Stadie, Zapp, and Lukens (127), and Stare and Baumann (131) though the effects found by these authors were smaller than those found by Krebs and Eggleston. Shorr and Barker found no insulin effect with any tissue other than minced pigeon muscle (chicken breast muscle, skeletal muscle of cat, dog, and rabbit, heart muscle of dog, and sliced pigeon muscle), though the other tissues all gave increased respiration when citrate and muscle juice were added. But Stare and Baumann (130), using Ringer-phosphate instead of plain phosphate buffer as medium, were able to produce some effects with chicken breast and rabbit heart and skeletal muscle. Stadie *et al.* did not find any increased activity of insulin *in vitro* with muscle from recently depancreatized pigeons or cats, but Stare and Baumann found the effect of insulin increased two or three times with muscle from pigeons depancreatized 1 to 3 weeks previously.[18] Stare and Baumann (131) found that the R.Q of minced pigeon muscle without additions fell from its initial high level to zero in the third or fourth hour of an experiment (the respiration also fell off greatly), while with insulin added, with or without added muscle juice and citrate or fumarate, the R.Q. was maintained near 1.0. Stadie *et al.* found the effect of insulin was no greater in the presence of citrate than in its absence; but Stare and Baumann, like Krebs and Eggleston, found that the effect of insulin, over the third and fourth hour of experiments, was increased in the presence of muscle juice with citrate, α-ketoglutarate, glutamate, or the C_4 acids. (Cocarboxylase addition caused a further increase in respiration.) Malonate inhibited the respiration in the presence or absence of insulin, and respiration was restored by fumarate,—citrate and glutamate were much less effective. Injection of malonate into rabbits was found to counteract somewhat the fall in blood sugar caused by insulin injection and to prevent convulsions. The insulin effect does seem to be connected with

[18] In the fourth week some of their birds recovered in health and the insulin effect almost disappeared. The pancreatectomy was not claimed to be complete; it is perhaps possible that regeneration of islet tissue occurred with the birds which recovered.

the activity of the citric acid cycle or the Szent-Györgyi scheme, but the mechanism of the effect is quite uncertain. It may be connected with the proper provision of oxidizable substrate.

d. *Remarks on the citric acid cycle, Szent-Györgyi's theory, and the cycle in kidney cortex slices.* In the light of Krebs' latest papers, it seems very probable that the citric acid cycle theory does describe the main method of carbohydrate oxidation in pigeon muscle and very likely in other tissues. Nevertheless the contradictory results of various authors indicate that a number of points remain to be settled. Several authors have found only small effects of citrate and Thomas finds the action of citrate is delayed compared with that of fumarate. It is possible that the condition of the birds used, with regard to nutrition and previous activity, may affect the *in vitro* behavior of pigeon muscle. Details of technique may also have varied in unnoticed but critical ways. Coenzymes, for instance, may be destroyed more readily under some circumstances than under others. Krebs pointed out that the rates of O_2 uptake found by some workers were often lower than his own. With liver, Elliott and Elliott were able to prove catalytic effects with the substances in question by studies of R.Q. and acid disappearance. But the respiration of the liver suspension fell off too soon to be able to show an oxygen uptake increase greater than the equivalent of the added substances. Liver suspension lost its activity rapidly, but intact excised liver could be kept for hours and still showed full activity when brought into suspension and tested at once.

It is not to be expected that the addition of any member of the cycle will always have the same immediate effect as any other. The rate of a complete "tour" of the cycle will depend upon the rate of the slowest individual reaction. The rate of citrate oxidation may not be the limiting factor while the rate of malate oxidation or citrate synthesis may be. In the latter case time may be required to build up the malate or oxaloacetate concentration sufficiently to give the maximum rate.

Citrate, in moderately high concentrations, often shows an inhibitory action. This may be due to inhibition of the citrate oxidizing system itself (see e.g., Bernheim, 24) or to a competitive inhibition of other oxidations resembling the effect of malonate. Inhibition by citrate may be sufficient to mask the catalytic effect until excess citrate has been removed. Further, it should be remembered that citrate (*iso*citrate) oxidation requires coenzyme II while malate oxidation requires coenzyme I. If a deficiency of coenzyme II is produced in the preparation of the tissue, time may be required to rebuild it from coenzyme I by

phosphorylation reactions (1). The observation of Banga *et al.* (14) that oxidation of pyruvate by dialyzed brain suspensions was less complete, i.e., less oxygen was taken up per molecule of pyruvate disappearing, when no adenylpyrophosphate was added to the mixture, suggests the possibility that a failure to produce coenzyme II by phosphorylation caused the cycle to be checked at the citrate stage.

Breusch and Stare and Baumann found that citrate was less effective than fumarate in increasing respiration in the presence of malonate. The tendency of added citrate to cause inhibition, added to the action of malonate, may be sufficient to depress the catalytic action of citrate. The inhibition by malonate is overcome when sufficient succinate accumulates to compete successfully with the malonate for the succinic dehydrogenase. But even before the succinate stage is reached, four oxidation steps, each involving an atom of oxygen, can proceed with fumarate, without hindrance by malonate, so that oxygen uptake can be appreciable from the start. On the other hand, only two oxidation steps after citrate can occur until a high enough succinate concentration is built up for the cycle to proceed further. α-ketoglutarate was more effective than citrate possibly because it is more rapidly broken down to succinate.

Krebs (79) was satisfied that the malate $\rightleftharpoons$oxaloacetate part of Szent-Györgyi's theory represented the main mediation of H transfer in the oxidation reactions of the citrate cycle, but he was not sure of the further mediation of succinate-fumarate. There is no doubt that this latter mediation can occur, and Krebs has shown it to be essential in the oxidation of various metabolites by *B. coli*. But, as was pointed out on page 279, the discovery of diaphorase makes it unnecessary to assume the mediation of succinate $\rightleftharpoons$ fumarate in all cases. The experiments of Banga (p. 275) in which the addition of yellow enzyme to the system caused increased respiration, might be explained as partly due to the yellow enzyme mediating direct malate oxidation through coenzyme. (The old yellow enzyme, flavin-nucleotide-protein, is related in structure and properties to diaphorase, flavin-adenine-dinucleotide-protein.)

It seems probable that distinct modifications of Krebs' cycle must occur in some tissues. The processes which take place in kidney cortex slices, described earlier, seem very similar to the citric cycle of muscle, and oxidations involved in the kidney cycle may also be mediated by oxaloacetate $\rightleftharpoons$ malate. But an essential difference is that the members of the cycle get rapidly used up in kidney cortex slices. The oxaloacetate formed in the cycle may be resynthesized to citrate, but this

must occur without the entry into the cycle of extra pyruvate molecules from outside the cycle. Whatever the process is, it probably occurs to some extent in various minced tissues, when an excess of the catalytic substances is added. Krebs and Eggleston (81, 82) found that added lactate or pyruvate was used up by minced muscle but had little effect on the rate of respiration during the first 30 to 60 minutes, presumably because the enzymes were kept nearly saturated with pyruvate formed from carbohydrate in the tissue. With liver suspension, Elliott and Elliott (46) found that addition of lactate and pyruvate increased the respiration immediately, as much as did citrate and the C_4 substances. This may mean that, in liver suspension at least, pyruvate can be rapidly transformed into the catalytic substances by a route not involving synthesis with a pre-formed sufficiency of oxaloacetate. The same holds for kidney slices, since, when pyruvate was added, more succinate accumulated in the presence of malonate than in its absence (47), yet the inhibition of succinate oxidation would cause little oxaloacetate to be available.

The citrate cycle, or a modification of it, is almost certainly not the the only process by which pyruvate is oxidized. A dismutation reaction of pyruvate, yielding acetic acid, is mentioned in the next section, acetic acid appears to be formed from pyruvate by various tissues (85, 100) and by bacteria, and it is well known that acetoacetate can be formed from pyruvate by liver (52, 3, 85, 86; see also p. 279).

As mentioned in a previous section, Elliott and co-workers found that, while the C_4 compounds were rapidly oxidized away by kidney cortex slices, with other sliced tissues tried, added fumarate had little effect on the respiration. Such negative results with sliced tissues do not disprove a catalytic rôle for the C_4 compounds. In sliced tissues the cells can probably retain their original supply of the C_4 substances. Added fumarate or malate may be oxidized to oxaloacetate to a small extent, but any accumulated oxaloacetate would inhibit further malate oxidation (see Banga, 11, 16; Green, 59), and the rate of respiration would continue to be limited by the rate at which oxaloacetate is removed by reduction or by synthesis to citrate. (See discussion by Greig *et al.*, 64.) It is possible that the respiration of slices and other tissue preparations may depend on this type of catalysis and yet not always respond to addition of the catalytic substances or malonate since, as Szent-Györgyi emphasized, the C_4 substances may be firmly bound to enzyme proteins in sufficient amount to carry all the respiration and not be rapidly displaced by malonate.

Finally, it should be borne in mind that the transamination reactions,

discussed later, may have important effects on the catalysis of tissue respiration by removing or producing α-ketoglutaric and oxaloacetic acids.

KETO-ACIDS—HYDROXY-ACIDS. The position of pyruvic acid as an intermediate in carbohydrate breakdown is now well known. Pyruvic and other α-keto-acids are also intermediates in protein metabolism through the oxidative deamination of amino-acids.

Tissue slices under aerobic conditions cause the oxidation of lactate primarily to pyruvate,[19] but added pyruvate becomes partly reduced to lactate (Elliott *et al.*, 49). Krebs and Johnson (85) and Elliott *et al.* pointed out that the lactate-pyruvate system may act as a mediator of hydrogen transport between systems which reduce pyruvate and the system which reoxidizes lactate.

Under anaerobic conditions, Krebs and Johnson found that slices of animal tissues, especially testis, metabolize pyruvic acid by means of a dismutation, yielding lactic and acetic acids and CO_2 according to the following equation:

$$2CH_3CO\cdot COOH + H_2O = CH_3CHOH\cdot COOH + CH_3COOH + CO_2$$

Elliott *et al.* simultaneously, but by different methods, also observed the dismutation with testis but found only slight anaerobic CO_2 evolution from pyruvate with other tissues.[20] Though all the tissues produced lactate from added pyruvate to some extent, this was probably due to reduction of pyruvate by other systems. The rate of anaerobic CO_2 evolution from pyruvate was not rapid enough, except with testis, to account for the whole rapid aerobic pyruvate removal as due to dismutation. Nevertheless it appears that various tissues can remove pyruvate by dismutation with itself.

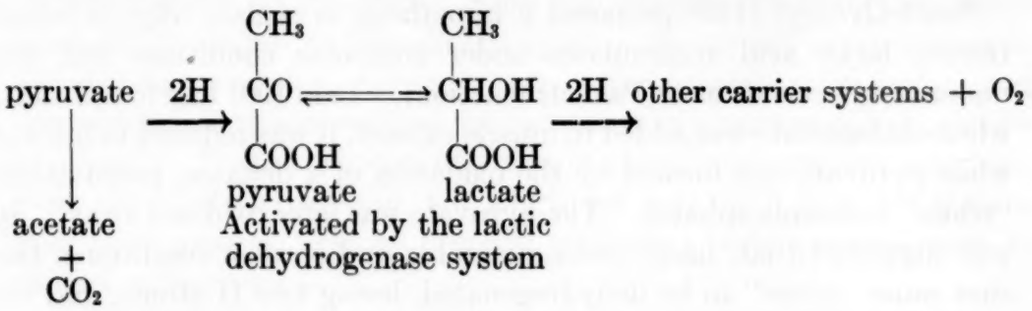
Krebs (77) found that *Gonococcus*, *Staph. aureus* and *albus*, and *Strept. faecalis* (but not a number of other bacteria) brought about a dismutation of pyruvate yielding lactate, acetate, and CO_2. He found that anaerobic CO_2 evolution was as great or greater than the aerobic and he concluded that the whole pyruvate metabolism occurred through the dismutation, and that the oxygen uptake observed aerobically was

[19] Greig *et al.* (64) have indicated that ox retina oxidizes added lactic acid to give pyruvic acid and some acid other than pyruvic or acetic. The unknown acid may have been formed in other tissues but differing relative rates of reaction may have prevented it from being apparent.

[20] The reason for the greater effects found by Krebs and Johnson is not clear. These authors used a bicarbonate-containing "saline" at 39–40° while Elliott *et al.* used the bicarbonate-containing medium of Krebs and Henseleit (83) at 37–38°.

due to reoxidation of the lactate produced. Barron and Lyman (21), however, showed that aerobically direct oxidation of pyruvate also occurred.

Probably the essential reaction in both dismutation and oxidation (other than oxidative syntheses giving citrate or acetoacetate) is an oxidative decarboxylation of pyruvate to acetate and CO_2; in dismutation other molecules of pyruvate act as primary hydrogen acceptor, in aerobic conditions oxygen is the ultimate hydrogen acceptor (see e.g., Lipmann, 99). Since lactate can be reoxidized to pyruvate, the pyruvate-lactate system may be regarded as a carrier for the oxidation of pyruvate itself. The system may be represented as follows:



Besides pyruvate, other keto-acids may act as carriers. In various sliced tissues, Krebs and Johnson (85) showed the occurrence of dismutative oxidations of pyruvate by acetoacetate and oxaloacetate, these substances being reduced to β-hydroxybutyrate and malate respectively. Further, α-ketoglutarate could be oxidized by acetoacetate giving succinate, CO_2, and β-hydroxybutyrate. Weil-Malherbe (150) showed the dismutation of α-ketoglutarate with itself in brain tissue, succinate, CO_2, and α-hydroxyglutarate being formed; evidence for the same reaction in kidney cortex was obtained by Krebs and Cohen (80).

All the reduction products of these dismutations,—lactate, β-hydroxybutyrate, α-hydroxyglutarate, and malate,—are known to be reoxidizable by tissues under aerobic conditions. The dismutations were shown to occur anaerobically. It is probable that they also occur in the presence of oxygen and that keto $\rightleftharpoons$ hydroxy-acid mediates aerobic oxidation of keto-acids. However, with pyruvate, and almost certainly with the other keto-acids, oxidation without this type of mediation also occurs in tissues.

It is probable that the carrier function of keto-hydroxy-acids is not limited to oxidations of keto-acids themselves. With isolated enzyme systems, dismutations have been demonstrated (Euler *et al.*, 53; Dewan and Green, 42; Green *et al.*, 60, Dewan, 41) whereby pyruvate and oxalo-

acetate accept hydrogen from triosephosphate, α-glycerophosphate, β-hydroxybutyrate and glutamate. The dismutation between pyruvate and triosephosphate is, of course, an essential step in the process of glycolytic lactic acid formation; the similar dismutation between acetaldehyde and triosephosphate which occurs in alcoholic fermentation should also be mentioned. The oxaloacetate-malate reaction is a part of Szent-Györgyi's scheme and, as discussed in previous sections, it is probably involved in a variety of oxidations; other keto-acids may play a similar rôle. An oxidation of citrulline by α-keto-acids, giving glutamic acid and urea, has been suggested as one of the mechanisms of urea formation in liver slices (Bach, 10).

Szent-Györgyi (140) proposed a hypothesis to explain why, in many tissues, lactic acid accumulates under anaerobic conditions but not under aerobic conditions (Pasteur reaction). Laki (90) had found that, when oxaloacetate was added to muscle extract, it was reduced to malate while pyruvate was formed by the oxidation of a donator, presumably "triose" (triosephosphate). The pyruvate was later oxidized away. It was suggested that, under both anaerobic and aerobic conditions, tissues cause "triose" to be dehydrogenated, losing two H atoms, and rearranged to give pyruvate. Anaerobically, pyruvate itself accepts the two H atoms giving lactate. Aerobically oxaloacetate takes up the hydrogen giving malate, but this is reoxidized by the fumarate-succinate-WKS-O_2 system, so that a supply of oxaloacetate is maintained. The pyruvate, aerobically, is oxidized away or re-synthesized to carbohydrate. Evidence was provided (Das, 40; Laki, 92) that oxaloacetate, if present, would accept the hydrogen more readily than would pyruvate. This scheme has been strongly supported by Parnas and Szankowski (115, see also 107).

GLUTAMIC AND ASPARTIC ACIDS. Among the products of protein metabolism, the two dibasic amino acids, glutamic and aspartic, have lately been shown to play a special part in oxidative metabolism.

Braunstein and co-workers (33, 31) found that a type of reversible oxidation-reduction process, in which a transfer of amino-groups as well as of hydrogen atoms occurs, takes place in most animal tissues, in plants, and in micro-organisms. This type of reaction has been called "transamination" and the following equations represent examples:

$$\underset{\text{glutamic acid}}{COOH \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH} + \underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} \rightleftharpoons$$

$$\underset{\alpha\text{-ketoglutaric acid}}{COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH} + \underset{\text{alanine}}{CH_3 \cdot CHNH_2 \cdot COOH}$$

$$\underset{\text{aspartic acid}}{COOH \cdot CH_2 \cdot CHNH_2 \cdot COOH} + \underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} \rightleftharpoons \underset{\text{oxaloacetic acid}}{COOH \cdot CH_2 \cdot CO \cdot COOH} + \underset{\text{alanine}}{CH_3 \cdot CHNH_2 \cdot COOH}$$

For transamination to occur, one of the reactants, the "primary substrate," must be a dicarboxylic α-amino-acid or α-keto-acid or a compound having similar electrostatic qualities, such as phosphoserine, cysteic, or homocysteic acid. The other reactant may be an α-amino- or α-keto-acid other than glycine; glycine, β- and γ-amino- or keto-acids, simple amines, aldehydes, ketones, and peptides, do not react. Transamination occurs selectively, and possibly exclusively, with the natural, *l*-series, amino-acids. Since the reactions are reversible, small amounts of amino- or keto-dicarboxylic acids can act as mediators in amino-group transfers between pairs of mono-carboxylic amino- and keto-acids. Transaminations are catalyzed by enzymes called "aminopherases". There are apparently two of these, since preparations have been obtained (Kritzmann, 88) which act only with glutamic acid or only with aspartic acid as primary substrate; both enzymes seem to require a coenzyme.

Euler, Adler *et al.* (54), in their studies on glutamic acid dehydrogenase, have shown that glutamic acid can be produced from α-keto-glutaric acid and NH_3 with dihydro-coenzyme (either coenzyme I or II with the enzyme from animal tissue) under the influence of the apodehydrogenase. The α-keto-glutaric acid and NH_3 reversibly and spontaneously give the imino-acid which is reduced by the dihydro-coenzyme. The glutamic acid formed, with aminopherase, can then transfer the amino-group to various α-keto-acids. Thus by the coöperation of glutamic dehydrogenase, aminopherase, a coenzyme-determined dehydrogenase, coenzyme, and a trace of glutamic or α-keto-glutaric acid, a synthesis of an amino-acid may be coupled with the oxidation of another substance. The following equations summarize the reactions involved:

$$\text{donator} + \text{coenzyme} = \text{oxidized donator} + \text{coenzyme } H_2$$

donator apodehydrogenase

$$\alpha\text{-ketoglutarate} + NH_3 = \alpha\text{-iminoglutarate} + H_2O$$

$$\alpha\text{-imino-glutarate} + \text{coenzyme } H_2 = \text{glutamate} + \text{coenzyme}$$

glutamic apodehydrogenase

$$\text{glutamate} + \alpha\text{-keto-acid} = \alpha\text{-keto-glutarate} + \text{amino-acid}$$

aminopherase

Net effect

$$\text{Donator} + \alpha\text{-keto-acid} + NH_3 = \text{oxidized donator} + \text{amino-acid} + H_2O$$

A similar series of reactions mediated by aspartic instead of glutamic acid is possible, particularly in plants; aspartic acid may also be produced from NH_3 and fumarate under the influence of aspartase (see 31).

Du Vigneaud and Irish (146), following earlier suggestions of Knoop, have indicated that amino-acids may be synthesized in the body from α-keto-acids and NH_3 by an oxidation-reduction reaction with pyruvic acid whereby CO_2 is liberated and the acetyl-amino-acid is formed. The latter is then hydrolyzed to acetic acid and the amino-acid.

Braunstein suggests that glutamic (or aspartic) acid with its aminopherase and dehydrogenase may be concerned in the oxidation of the natural, *l*-series, amino-acids. Since the work of Bernheim and Bernheim (25) and Krebs (75), the presence in tissues of an enzyme which causes the oxidative deamination of *d*-amino-acids has become well known; this enzyme belongs to the group of flavin-adenine-dinucleotide-protein complexes. But the system responsible for the oxidation of the natural amino-acids appears to be much more complex and labile; no simple *l*-amino-acid oxidizing enzyme, other than glutamic dehydrogenase, can be separated from animal tissues. Braunstein suggests that the *l*-amino-acids undergo transamination with α-keto-glutarate yielding the α-keto-acids and glutamic acid; the latter is then reoxidized to α-keto-glutarate by glutamic dehydrogenase, with the liberation of NH_3. The reactions involved may be represented as follows:

$$\text{l-amino-acid} + \alpha\text{-keto-glutarate} = \alpha\text{-keto-acid} + \text{glutamate}$$
aminopherase

$$\text{glutamate} + \text{coenzyme} = \text{imino-glutarate} + \text{coenzyme}\cdot H_2$$
glutamic apodehydrogenase

$$\text{coenzyme}\cdot H_2 + O = \text{coenzyme} + H_2O$$
diaphorase and the cytochrome system (probably)

$$\text{imino-glutarate} + H_2O = \alpha\text{-keto-glutarate} + NH_3$$

Net effect

$$\text{l-amino-acid} + O = \alpha\text{-keto-acid} + NH_3$$

Braunstein (32) has actually set up an artificial mixture of enzymes, coenzymes, and α-keto-glutarate, which will bring about the oxidation of certain *l*-amino-acids.

Virtanen and Laine (147) have indicated that in root nodules of leguminous plants, hydroxylamine, produced in the fixation of nitrogen, may combine with oxaloacetic acid to give oximinosuccinic acid which is reduced to aspartic acid. The aspartic acid may then transfer the amino-group to the other α-keto-acids giving rise to amino-acids.

Braunstein (31) suggests a number of other ways in which transaminations may affect respiration and metabolism.

The extent to which such processes as those described above can take place in various tissues depends on how many of the amino- or keto-acids can take part in transaminations. Cohen (36) found that, in pigeon breast muscle, out of 21 amino-acids tried, only aspartic acid and alanine react rapidly with α-keto-glutarate; valine and α-amino-butyric acid reacted slightly. Of a series of α-keto-acids, only oxalo-acetic and pyruvic acids reacted rapidly with glutamic acid. However, in liver and kidney cortex Cohen (37) has found much wider reactivity. In liver appreciable reaction occurred between α-keto-glutaric acid and aspartic acid, alanine, arginine, iso-leucine, leucine, valine, phenyl-alanine, and histidine. Kidney cortex was active with all these, except histidine, and also with cysteine, methionine and lysine. No reaction occurred with glycine, proline, tryptophane, or any *d*-amino-acid.

Krebs and Cohen (80) found with kidney cortex slices or minced heart muscle, but not appreciably with other tissues, that added α-keto-glutarate and NH_3 undergo the following reaction:

$$2\alpha\text{-keto-glutarate} + NH_3 = \text{glutamate} + \text{succinate} + CO_2$$

They considered that the reaction was essentially a dismutation between α-iminoglutarate, formed from α-keto-glutarate and NH_3, and unchanged α-keto-glutarate, as follows:

$$\alpha\text{-iminoglutarate} + \alpha\text{-keto-glutarate} + H_2O = \text{glutamate} + \text{succinate} + CO_2$$

Since glutamate can be reoxidized to α-iminoglutarate, the system α-iminoglutarate ⇌ glutamate may act as a hydrogen transporting system. It had been observed previously (Krebs, 74; Edson, 44) that addition of NH_4Cl catalytically increased the respiration of kidney cortex in the presence of α-keto-glutarate or substances, such as lactate, pyruvate, or glucose, which might give rise to α-keto-glutarate.

Other systems. *Formate-bicarbonate.* Krebs (76; see also 109; 39) found that *B. coli.*, in bicarbonate-containing medium under anaerobic conditions, brings about the reaction:

$$\underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2O = \underset{\text{acetic acid}}{CH_3\cdot COOH} + \underset{\text{formic acid}}{HCOOH}$$

He considered that this reaction takes place in the following manner:

$$CH_3\cdot CO\cdot COOH + O \mid H_2 + CO_2 = CH_3\cdot COOH + CO_2 \mid + HCOOH$$

The CO_2 or bicarbonate of the medium accepts hydrogen, giving formate. Since, under aerobic conditions, *B. coli* causes the oxidation of

formate to CO_2 (bicarbonate), the formate-bicarbonate system may act as a carrier in the oxidation of pyruvate and other substances in certain organisms. There is no evidence for such a possibility in animal tissues.

Catechol-o-quinone. It is probable that the polyphenol oxidase-polyphenol system performs in certain plant tissues a similar function to that of the cytochrome oxidase-cytochrome system. Long ago, Palladin (114) suggested that the oxidation products formed by polyphenol oxidase (catechol oxidase) action oxidize cell substances. Kubowitz (89) observed that o-quinone, formed by the action of catechol oxidase on catechol, can oxidize the dihydro-coenzymes I and II, and this shows that catechol-o-quinone, with catechol oxidase, can act as a hydrogen transporting system for all the coenzyme-determined dehydrogenase systems. Peroxidase and H_2O_2 also oxidize phenols so that the catechol-o-quinone system can mediate oxidations by H_2O_2 when peroxidase is present.

Catechol and derivatives of it are widely distributed among plant tissues. Boswell and Whiting (29) obtained evidence that as much as 66 per cent of the respiration of potato slices was catalyzed by the catechol system.

Ascorbic acid. Ascorbic acid oxidation can be catalyzed by ascorbic oxidase (139, 143), by o-quinones (138, 69, 72), by cytochrome-cytochrome oxidase (138, 19), and by Cu and other compounds (138, 133, 20). Tissues reduce oxidized ascorbic acid (dehydro-ascorbic acid) rapidly and ascorbic acid may serve some function as mediator of oxidations. However, no dehydrogenase systems are known to reduce dehydro-ascorbic acid, though glutathione and protein-SH groups can do so (138, 28, 121, 68). Szent-Györgyi (139) indicated that the ascorbic acid system plays an important part in mediating the respiration of cabbage leaves.

Dihydroxymaleic acid. Banga and Szent-Györgyi (18) discovered that plant tissues contain an enzyme which causes the vigorous oxidation of the C_4-dicarboxylic acid, dihydroxymaleic acid. The oxidation of this substance was also catalyzed by inorganic iron or copper. The substance could also be oxidized indirectly by oxygen, or by H_2O_2 with peroxidase, through certain phenol-quinones (Robeznieks, 120; Philippot, 116). The oxidation product, diketosuccinic acid, was believed to be re-reduced by tissue systems and so may act as a mediator of respiration (Banga and Philippot, 15, Philippot 116).

Adrenochrome. Adrenaline can be oxidized by the cytochrome-

cytochrome oxidase system, or by catechol oxidase, to adrenochrome,[21] N-methyl-2,3-dihydro-3-hydroxyindole-5,6-quinone (Green and Richter, 61). Green and Richter found that this red pigment could accept hydrogen from reduced coenzyme I and the leuco-adrenochrome formed could then be reoxidized either by the cytochrome-cytochrome oxidase system or by another, cyanide insensitive, system which was not further studied. In this way adrenochrome could mediate oxygen uptake with a number of coenzyme-dehydrogenase systems. From the activity *in vitro* of low concentrations of adrenochrome, Green and Richter concluded that the carrier function of this hormone derivative might be of physiological significance.

Stickland reaction. Stickland (132, see also 151, 56) discovered that the anaerobic organism, *Clostridium sporogenes*, can produce energy for growth by the oxidative deamination of one group of amino-acids while certain other amino-acids act as hydrogen acceptors.

CONCLUSION

Until quite recently studies on tissue respiration and the mechanisms which promote biological oxidation-reduction reactions, have been carried on rather independently of other fields of chemical physiology. The work described above has shown that the fields of intermediary metabolism and respiratory catalysis cannot be separated, since substances which are known to be intermediate products of the breakdown of foodstuffs are also parts of the catalytic mechanisms for the energy-yielding oxidations of such foodstuffs.

The synthesis of amino-acids from keto-acids and NH_3, and the breakdown of the *l*-amino-acids may involve the mediation of the dibasic amino-acid-keto-acid system and coupled oxidation or reduction of other substances. Work of the last few years has shown that various oxidation-reductions are coupled with phosphorylation of glucose and other compounds; as a result, studies of tissue oxidations have become connected with the study of glucose and glycogen synthesis and breakdown, and various other processes which involve phosphoric acid esters. The discovery of the coupling of phosphorylation with oxidation-reduction has provided an understandable mechanism whereby the energy of oxidations is made available for certain syntheses.

Tissue respiration studies are now intimately connected with the

[21] Adrenaline can also be oxidized by animal tissue preparations to give, primarily, 3,4-dihydroxy-phenyl-glycollic aldehyde, through the action of adrenaline or amine oxidase (Blaschko et al., 27).

science of nutrition, since several of the B group of vitamins are known to be parts of the prosthetic groups of oxidation enzymes. The field of endocrinology is impinged upon since a definite effect of insulin on respiration is now known, a carrier function for adrenochrome is suspected, and an oxidative enzyme (amine oxidase) causes the inactivation of adrenaline. Proteolytic enzyme chemistry is connected to oxidation-reduction chemistry through the action of glutathione, ascorbic acid, and other reversibly oxidizable systems, on the —SH groups which are necessary for the activity of some proteolytic enzymes.

More than ever biochemists need to have the "biological outlook," the recognition of the complex interlocking of dynamic systems in biological materials. One aspect of this interlocking has been outlined in the preceding pages.

Appendix. Since this review went to press several further papers concerning the citric acid cycle have appeared.

Smyth (156), in Kreb's laboratory, has shown the rapid oxidative utilization of pyruvate by minced sheep heart. He applied the same type of experimentation as was used by Krebs and Eggleston (82) with pigeon breast muscle (p. 287) and obtained very similar results. He concluded, in agreement with Hallman and Simola (66), that the citric acid cycle plays a part in the metabolism of heart tissue. He noted that citrate, especially in raised concentrations, has a smaller effect on the respiration than the other substances concerned in the cycle and occasionally even increased the inhibition with malonate.

Evans (152), also in Kreb's laboratory, has shown that in minced pigeon liver, oxaloacetate and pyruvate react to form citrate and α-ketoglutarate and added citrate is oxidized, indicating that the citric acid cycle can occur in liver. But in the presence of added pyruvate, the oxygen uptake and the very rapid pyruvate utilization are not inhibited by malonate, and the addition of fumarate with or without malonate has no effect. With high concentrations (0.02–0.04 M) of pyruvate, an accumulation of α-ketoglutarate occurs, and, in the presence of malonate, acetoacetate and succinate also accumulate. The formation of acetoacetate from pyruvate by liver mince has been shown previously (see pp. 279 and 293). The formation of α-ketoglutarate in liver evidently does not require the addition of C_4 dicarboxylic acid (see also p. 293). But α-ketoglutarate and citrate are formed from oxaloacetate plus pyruvate anaerobically much more rapidly than from pyruvate alone. It is therefore probable that, at low concentrations, pyruvate is metabolized largely by way of the citric acid cycle.

Krebs *et al.* (154) found that guinea-pig kidney, as well as pigeon breast muscle and sheep heart, with added oxaloacetate in anaerobic conditions, gives CO_2, fumarate, malate, α-ketoglutarate, citrate and succinate in the amounts to be expected from the citric acid cycle theory with oxaloacetate acting as hydrogen acceptor. The oxaloacetate also provides, by its own decomposition some of the pyruvate necessary for the citrate synthesis.

In sheep brain and testis, Krebs *et al.* found citrate and α-ketoglutarate formation from oxaloacetate suggesting the occurrence of the cycle or some of its

component reactions also in these tissues. But no conclusion could be drawn as to the quantitative importance of these reactions in brain and testis in view of the results obtained by Banga *et al.* (14) with brain (p. 286) and by Krebs and Johnson (85) and Elliott *et al.* (49) with testis (p. 294).

Using bicarbonate containing labelled, radioactive, carbon, Evans and Slotin (153) have shown that carbon dioxide in the medium participates in the synthesis of α-ketoglutarate from pyruvate in pigeon liver. Presumably the carbon dioxide combines with pyruvate giving oxaloacetate which then combines with more pyruvate to give α-ketoglutarate via citrate. This observation would explain Evans' observation, mentioned above, that the formation of α-ketoglutarate by liver does not require the addition of C_4 dicarboxylic acid. Krebs (155) also mentions that he has evidence for the carboxylation of pyruvate in pigeon liver. He states that experiments on vitamin B_1 deficient pigeons suggest that the reaction between carbon dioxide and pyruvate is the stage at which the vitamin enters the metabolism of pyruvate in animal tissues and that the vitamin is not concerned with the direct oxidation of pyruvate.

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THE EFFECT OF ANOXIA ON THE ALIMENTARY TRACT

EDWARD J. VAN LIERE

From the Department of Physiology, School of Medicine, West Virginia University, Morgantown

The effect of anoxia on respiration, on the blood and circulation, and on the central nervous system has interested physiologists for over a half-century. Studies on the effect of anoxia on the alimentary tract, however, relatively have been neglected. One reason for this may be that the effects of anoxia on the alimentary tract are not manifested as strikingly as on other organs or systems of the body; moreover, observations on the gastrointestinal tract are more difficult to make since cumbersome apparatus is often needed which precludes making observations at high altitudes.

It is known that the effect of anoxia depends upon the length of sojourn at high altitudes and on the degree of the severity of the anoxia. In this review the degree of anoxia will be expressed as partial pressures of oxygen in millimeters of mercury, and in order to help orient the reader the approximate altitude in feet will also be given.

Since the oxygen percentage composition of the atmosphere remains practically unaltered, at least to a height of 72,000 feet (39), it is confusing to express altitude in percentage of oxygen. The latter expression may be used, however, if a known percentage of oxygen is delivered at a given concentration by some mechanical device. The degree of anoxia may also properly be expressed by stating the actual barometric pressure.

In this review, four types of anoxia will be recognized: 1, the anoxia type; 2, the anemic type; 3, the stagnant type; 4, the histotoxic type.

Effect on motility. As far as the author is aware, no work has been reported in the literature on the effect of anoxia on swallowing movements. The mechanism is a complicated one and it is doubtful whether the normal act of swallowing is entirely understood. No work, furthermore, has been reported on the effect of anoxia on the esophagus or on the cardia. Most of the work dealing with the effect of anoxia on the movements of the gastro-intestinal tract has been done on the stomach; some work also has been reported on the pyloric sphincter.

EFFECT OF ANOXIC ANOXIA ON GASTRIC MOTILITY. *Hunger contractions.* In 1928 Van Liere and Weaver (unpublished work) and later Van Liere and Crisler (42) using the balloon method, showed that anoxia decreased the amplitude of hunger contractions in the normal trained dog. At a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) or even at a somewhat higher partial pressure of oxygen there was a distinct loss of gastric tone and a diminution in the height of the hunger contractions; they still persisted faintly, however, at a partial pressure of oxygen of 40 mm. of Hg.

Hellebrandt, Brogdon and Hoopes (20) in 1935 studied the effect of acute anoxia on hunger contractions in man. Their subjects were allowed to breathe 8 per cent oxygen or less from 5 to 14 minutes out of a closed system; the carbon dioxide was removed by soda lime. These authors reported that although motility seemed more susceptible to anoxia than secretion, the degree of change was not marked. They concluded that the pre-coma type of anoxia had but little effect on hunger contractions. Krugly (21) in 1938, working with dogs, reported that hunger contractions were inhibited at a barometric pressure from 320 to 357 mm. Hg.

Motility of the stomach containing food. Using both the balloon method and the gastrograph, Crisler, Van Liere and Booher (8) working with barbitalized dogs, found that a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) or somewhat less, caused inhibition of gastric digestive motility. There was a decreased amplitude of contraction and a fall of gastric tone. At a partial pressure of oxygen of 50 mm. Hg the digestive contractions were greatly diminished in height but were not abolished. No retching or vomiting movements were reported. The authors felt that the most plausible mechanism for the early inhibition of digestive contractions produced by anoxia was a sensitization of the sympathetics by the rise in pH accompanying the initial hyperpnea and hyperventilation in anoxia.

Hellebrandt, Brogdon and Hoopes (20) worked on the effect of acute anoxia on digestive contractions in the human using the same technique and same percentages of oxygen as was used in their work on hunger contractions. They reported that anoxia moderately depressed the motor activity of the stomach, but concluded that the pre-coma type of anoxia had relatively little inhibitory effect upon digestive motility. Since these authors used an entirely different technique from that reported by Crisler, Van Liere and Booher, the results obtained by the two different groups of workers are not incompatible.

Effect on motility of gizzards of birds. Nolf in 1925 published several papers (25, 26, 27) on the influence of hypercapnia and anoxia on the motility of the gizzards of birds. He found that by stimulating the extrinsic nerves of the gizzard for a few seconds, contractions were set up which continued for about 15 minutes. If the animals were subjected to an 8 or 10 per cent oxygen mixture in hydrogen or to air or hydrogen mixed with 5 per cent carbon dioxide, the contractions of the gizzard would be greatly depressed or even inhibited. As soon as the birds were removed from the influence of anoxia or the carbon dioxide mixture, the contractions of the gizzard would be resumed. He further showed that the adrenals played a part in inhibiting the contractions produced by anoxia; while extirpation of these organs did not abolish the effects of anoxia, the response was slower after their removal.

Effect on gastric emptying time. In 1933 Van Liere, Crisler and Robinson (43) reported that dogs subjected to anoxia showed a delay in the emptying time of the stomach. At a partial pressure of oxygen of 117 mm. of Hg (8,000 ft.) three of four dogs showed a delay in gastric emptying as determined by the fluoroscope. The authers considered this degree of anoxia a threshold for the normal dog. The more severe the degree of anoxia the greater was the prolongation of the empyting time of the stomach. At a partial pressure of oxygen of 73 mm. of Hg (20,000 ft.) the 2 dogs subjected to this degree of anoxia still had food in their stomach at the end of 24 hours. Later work (unpublished) on 23 additional dogs fully corroborated the work reported previously which was based on 6 animals. Krugly (21) in 1938 also reported that gastric emptying in dogs was prolonged by oxygen want.

In 1936 the author assisted by Lough and Sleeth (47) reported work on the effect of anoxia on the emptying time of the human stomach. Eight young male adults were used as subjects. Anoxia was produced by placing the subjects in a low-pressure chamber (41) and gastric emptying was determined by use of the fluoroscope. The work with human beings correlated well with that found in the dog although the dogs appeared to be somewhat more susceptible to anoxia. At a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) 2 of 3 subjects showed a slight but definite prolongation of gastric emptying. The authors gave this as the probable threshold for the average unacclimatized individual. At a partial pressure of oxygen of 94 mm. Hg (14,000 ft.) all of the 8 subjects showed a delay in gastric emptying time. Marked individual variations were noted; the range varied from 13.2 per cent to 166.9 per cent. Two of the individuals were subjected to a

simulated altitude of 18,000 feet. The authors concluded that with man, as in case of the dog, the more severe the degree of anoxia the greater the delay in gastric emptying.

Anoxia produced by the administration of nitrous oxide anesthesia. Sleeth and Van Liere (36), making a comparative study of the effects of various anesthetic agents on gastric emptying in the dog, found that nitrous oxide caused a delay in gastric emptying of 15 per cent. Their interpretation was that the delay was produced principally by anoxia. Since in administering nitrous oxide it is necessary to use a concentration of 95 per cent, the animals were actually getting 5 per cent oxygen during the 15 minutes the anesthesia was given.

Mechanism of the delay in gastric emptying produced by anoxic anoxia. Crisler, Van Liere and Wiles (9) working with dogs came to the conclusion that the delay in gastric emptying produced by anoxic anoxia was on a vagospastic pylorospastic basis, that is, the anoxia stimulated the vagus causing some contraction of the pyloric sphincter and this narrowing of the pyloric passage delayed stomach emptying. Anoxia produced this pylorospasm until a critical threshold was reached; beyond this threshold further delay was caused by the oxygen want acting directly on the smooth muscle of the stomach. The authors believed that the same factors were operative in man as in the dog.

Another mechanism must also be considered. If the secretion of epinephrine is capable of being augmented by anoxia, the increased epinephrine secretion could cause a definite inhibition of gastric motility. It has been shown in human beings (48) that ephedrine greatly delays gastric emptying. It is also known that epinephrine may produce a delay in gastric emptying time (37, 59).

Effect on the pyloric sphincter. In 1935 Van Liere, Crisler and Wiles (44), working with barbitalized dogs and using a pressure tonometer in the pylorus as described by Thomas (40), studied the effect of anoxic anoxia on the pylorus. A variability in the results was reported which they attributed to the complexity of the control of the pylorus and to the general nature of the stimulus of anoxia. Anoxia caused a rise in the tone in the pyloric sphincter in some animals and a fall in others. The height of the pyloric contractions, however, was generally diminished, especially if severe grades of anoxia were used.

Later studies (53), using a pressure tonometer in the pylorus of unanesthetized dogs with permanent gastric and duodenal fistulae, demonstrated that anoxia produced a rise in the tone of the pyloric sphincter in 14 out of 17 trials. The threshold for the production of this increase of tone in the unanesthetized dog was a partial pressure of

oxygen of 108 mm. Hg (10,000 ft.). The effect of the anoxia on the pyloric contractions was indeterminate. Concomitant tracings were taken of the pyloric antrum; anoxia decreased the tone of the pyloric antrum and effective grades of anoxia abolished the normal rhythmical contractions.

EFFECT OF STAGNANT ANOXIA ON GASTRIC MOTILITY. This type of anoxia may be caused by cardiac failure, impaired venous return, or by shock. While it is known clinically that certain cardiac diseases may produce nausea and vomiting as well as minor gastric disorders, it is difficult to find in the literature any quantitative data or any carefully controlled work on the effect of stagnant anoxia on gastric emptying. In order for the work to be well controlled, it would be necessary to know what the normal gastric emptying time of the patient had been before the stagnant type of anoxia had developed. Other difficulties would present themselves, such as the psychic factor in cardiac disease, the relatively physical inactivity of the patient, and others. Since any of these factors could influence gastric emptying, it would be necessary to evaluate very carefully any results obtained.

EFFECT OF ANEMIC ANOXIA ON GASTRIC MOTILITY. Carlson (6) in 1918 reported work on the effect of acute hemorrhage on hunger contractions. After bleeding two dogs about 30 per cent of their calculated blood volume, he found that the hemorrhage induced temporarily a greater gastric tonus and intensity of hunger contractions than those typical for these dogs before the hemorrhage. This augmented effect disappeared in less than 24 hours. Carlson felt that the tissues deprived of so much nutritive material possibly liberated a hormone which acted on the neuromuscular apparatus of the stomach stimulating hunger contractions. No subsequent proof, however, has been offered for this interpretation.

The effect of acute hemorrhage on gastric motility has been studied in human beings and in dogs by Van Liere, Sleeth and Northup (52). They reported that in the human being, after one-tenth of the calculated blood volume had been withdrawn, the gastric emptying time was prolonged an average of 41 per cent in 4 individuals. In no case was it less than 25 per cent. There was still a delay from 15 to 20 per cent in three of the men 24 hours after the hemorrhage. The stomach had apparently regained its normal motility in all four subjects at the end of 48 hours. Two dogs which too had one-tenth of their calculated blood volume withdrawn, also showed a noticeable delay in gastric emptying thus confirming the results obtained in man.

In 1938 Curtis and Hamilton (10), using the balloon method for ob-

serving gastric motility, reported studies made on patients suffering from pernicious anemia. Prior to the administration of liver therapy, an intense and persistent gastric motility was observed. This persisted during liver therapy and continued even after marked clinical improvement was noticed in the patient. The authors could not account for this hypermotility but suggested that it might have been produced by the associated anacidity or possibly by stimulation of the gastric neuromuscular apparatus by pathological changes in the stomach. These authors, using the same method, also reported continuous motility with frequent contractions of high amplitude in a case of hypoplastic anemia.

No well controlled work could be found in the literature of the effect of a primary anemia or a chronic secondary anemia or the leukemias on the gastric emptying time.

Carbon monoxide poisoning. Carbon monoxide exerts its action mainly by uniting with the hemoglobin of the blood and in this way prevents the hemoglobin from transporting the normal amount of oxygen. It therefore produces an anemic type of anoxia. It may, however, also act as a histotoxic agent. Peterson, Smith, and Hale (30), working with rats, reported that chronic carbon monoxide poisoning inhibited gastro-intestinal peristalsis.

EFFECT OF HISTOTOXIC ANOXIA ON MOTILITY. The cyanides exert the greatest depression on cellular oxidation. They are not, however, widely used in medicine and no reports could be found in the literature of the effect on gastro-enteric motility in the intact animal of cyanides or any other agent unequivocally producing histotoxic anoxia. Alvarez (1), however, has reported that the more highly active portions of the isolated strips of small intestine are inhibited more by cyanides than are less active strips.

There is a difference of opinion as to the degree of histotoxic anoxia produced by the inhalation anesthetics during surgical anesthesia. According to Dr. R. M. Waters (56) general anesthetic agents even when surgical anesthesia is produced, should not, if properly given, produce anoxia. Since all general anesthetic agents, however, produce respiratory depression in some measure, anoxia, unless the anesthesia is carefully given, may be produced by these agents. Anoxic anoxia is particularly difficult to avoid when nitrous oxide is the sole anesthetic agent.

A comparative study of common inhalation anesthetics has been reported on gastric motility in dogs (36). Surgical anesthesia (third plane) was rapidly induced and maintained for 15 minutes. As soon

as the animals regained consciousness they were allowed to eat the test meal. Their normal emptying had been determined previously. Fluoroscopic examination showed that all the anesthetic agents used prolonged gastric emptying time: chloroform by 64 per cent; ether by 40 per cent; nitrous oxide by 15 per cent; ethylene, cyclopropane, and divinyl oxide each by about 7 per cent. The severe anoxia accompanying the nitrous oxide anesthesia probably explains the longer delay with nitrous oxide than with the other gases. Emerson (13), using Macht's technique, found that surgical anesthesia with ether inhibits the motility of the small intestine of rats for the period of its duration. The intestine regained approximately its normal activity by the second hour after anesthesia was terminated.

These results have been interpreted by some authors as being due to sympathetic stimulation but if later work shows that these anesthetic agents exert a histotoxic effect in surgical anesthesia, their action could be explained on this basis.

With the fixed narcotics, effects directly on smooth muscle or on its innervation as a rule outweigh any possible histotoxic anoxia produced.

EFFECT OF ANOXIA ON THE MOVEMENTS OF THE SMALL INTESTINE AND COLON. It can be demonstrated easily that fulminating anoxia may stimulate intestinal peristalsis. If a guinea pig be struck a blow on the head and the abdomen be opened, the small intestines in many instances can be seen to undergo an active peristalsis. This initial stimulation apparently produced in part by the anoxia is, of course, but transitory in nature and is soon followed by a paralysis of the whole intestine.

In 1934 Schnohr (33) studied intestinal peristalsis by inserting an oval cellophane window in the abdominal wall of rabbits. He reported that anoxia or an increase in carbon dioxide concentration in the blood caused violent contraction of the arteries of the intestine and an immediate cessation of all intestinal movement.

The work of Emerson (13) on the effect of ether surgical anesthesia on the motility of the small intestine has been mentioned under the discussion of histotoxic anoxia. Peterson, Smith, and Hale (30), whose work was mentioned when CO poisoning was discussed, used a modification of Macht's technique in studying the effect of CO on gastrointestinal motility in rats. They found that if the blood be saturated from 70 to 80 per cent with CO, gastro-peristalses were inhibited 33 per cent. What they termed, "the egestion time", that is, the time required for the first appearance of fecal pellets following the test meal, was prolonged 22 per cent.

The results obtained by the various workers on the effect of anoxia on

the motility of the small intestine are not unexpected. As far as it is known, the innervation of the small intestine is similar to that of the stomach and it is likely that anoxia would have similar effect on both organs; the difference, if any, would be one of degree.

No quantitative data could be found in the literature on the effect of anoxia on the intact colon alone. The colon has a different innervation from that of the stomach or small intestine and it would be unsafe to predict what effect anoxia would have on its movements. It is believed by some (14) that the blood supply of the colon is much less rich in oxygen than is that of the upper part of the gastro-intestinal tract. If this is true, it may be that the colon is relatively resistant to oxygen want. This, however, needs further study.

Since it is necessary to subject both man and animals to a pronounced degree of anoxia before the movements of the stomach are significantly affected, it may be inferred that the nausea and vomiting produced by high altitudes are not due to a direct effect on the stomach. It is known that some people suffer from marked mountain sickness at an altitude of 10,000 feet. At this altitude no pronounced change is noted in gastric motility.

Aviators often state that flying at high altitudes causes them to be hungry. It may be that these men confuse hunger with appetite. It is, of course, possible that in man certain altitudes may cause slight stimulation of hunger contractions. The carefully controlled work on dogs, however, gave no indication of this.

As previously mentioned, studies on the effect of anoxia on gastric emptying show a good deal of individual variation in both man and dog. Individuals who are particularly sensitive to oxygen want would show a pronounced retardation of gastric emptying at the altitude, for example, that a transcontinental airplane flies, that is, about 12,000 feet. Less susceptible individuals would be much less affected.

It must be recognized, however, that a moderate diminution of gastric motility presumably is of little practical significance. Anoxia doubtless would cause cerebral manifestations and cardiac dysfunction before any significant impairment would be noted in the behavior of the stomach. Compared to the central nervous system, the stomach is relatively resistant to anoxia.

ANOXIA AND GASTRO-INTESTINAL PERMEABILITY. The work of Hamburger (18) on the intestine, Bainbridge (4) on the permeability of the visceral capillaries, Starr (38) on the kidney, Landis (22) on the capillaries of the mesentery of the frog, Magee and Macleod (23) on

the intestine, and Van Liere and his co-workers (45) also on the intestine indicate that anoxia is capable of influencing the permeability of epithelium.

The difficulty with most of the work reported, however, is that the degree of anoxia was not measured. In some instances anoxia was produced by shutting off the blood supply or by producing injury to the epithelium by the application of corrosive chemical agents. Shutting off the blood supply produces a rapid and complete anoxia leading to irreparable injury in a short time; chemical injury to cells produces a dead membrane rather than a normal semi-permeable one. Conclusions drawn from experiments so performed must be accepted with some hesitation.

In order to avoid some of the criticism just offered, the following procedure has been used by the author and his co-workers in studying the effect of anoxia on absorption from the intestine of various substances. Matched pairs of dogs which had had no food 48 hours previous to the experiment were used. Sodium barbital (220 mgm. per kgm. body weight) given intravenously was used for an anesthetic. One animal served as a control and the other was subjected to anoxia. The small intestine was washed out with an isotonic solution of either sodium chloride or glucose heated to body temperature. With the exception of the duodenum, the entire small intestine was used as a loop. The loops were made of the same length by actual measurement. The substance to be studied was brought to body temperature before being placed in the intestine and was left in long enough so that at least 50 per cent was absorbed. Undue distention of the intestine was avoided. At the end of a given time the contents of the loops were removed and carefully measured and analyzed quantitatively for the substance in question. In nearly all the studies varying degrees of anoxia were used, ranging from a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) to that of 53 mm. Hg (28,000 ft.) which partial pressure is about as low as is compatible with the life of a barbitalized animal over a period of two or three hours.

EFFECT OF ANOXIA ON ABSORPTION. *Absorption of water.* Bainbridge (4) in 1906 in explaining some of the factors concerning the flow of post mortem lymph suggested that lack of oxygen increased the permeability of visceral capillaries. It has been shown that the glomerular filtrate in a kidney with normal circulation is protein free (Wearn and Richard, 1924) (57). Starr (38) in 1926 showed, however, that a transient albuminuria not accompanied by any microscopically visible pathological

change could be produced by a stoppage or even a reduction of blood flow.

Landis (22) in 1928 studied the effects on capillary permeability of oxygen, high tensions of carbon dioxide and increased hydrogen ion concentration in single capillaries of the frog mesentery. He found that after a three minute period of oxygen lack, which he produced by compressing the mesenteric artery and vein, the permeability was so increased that the fluid in the capillary filtered through the wall at approximately four times its normal rate. Protein also passed through the capillary wall so that the effective osmotic pressure of the plasma proteins was reduced to one-half their normal value. As soon as the circulation was allowed to return, the capillary wall rapidly recovered its impermeability. When the mesentery was exposed to Ringer's solution completely saturated with carbon dioxide, the capillary permeability was slightly increased to fluid but remained normally impermeable to protein; one-half saturation of carbon dioxide had no effect. Within physiological limits an increase in hydrogen ion concentration produced practically no change in capillary permeability.

Van Liere, David and Lough (45) in 1935 reported studies on the effect of various degrees of anoxia on abosrption of water from the small intestine of barbitalized dogs, using the procedure previously outlined. They reported that absorption of water was not affected by anoxia until a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) was reached. At this partial pressure of oxygen, considerably more water was absorbed by the anoxic dogs than by the controls. The results when statistically analyzed were highly significant. At partial pressures of oxygen of 63 and 53 mm. of Hg (24,000 and 28,000 ft.) the results, although not as striking, showed a definite trend in the same direction, that is, the dogs subjected to anoxia absorbed more water. These experiments were performed on a large number of dogs and it may be said then that the most rapid absorption of water from the small intestine of the mammal takes place at a partial pressure of oxygen of 80 mm. Hg. No adequate explanation can be offered for this rapid absorption of water at this particular pressure of oxygen. It is of interest to note, however, that this occurred at what Barcroft has termed the critical level of anoxia.

Van Liere, Northup and Sleeth (49) working with barbitalized dogs and using virtually the same technique as previously outlined, found that ephedrine administered either orally or intravenously had no effect on the intestinal absorption of water. When ephedrine was given intravenously during the period of absorption, the amount of water absorbed

from the small intestine was very slightly decreased. These findings are of interest since it was shown by Rudolf and Graham (32) that ephedrine produces vasoconstriction of the blood vessels of the intestines.

It has been shown (50) that in anemic anoxia less distilled water was absorbed from the small intestine of dogs which had suffered a hemorrhage of 3.2 per cent of their body weight. Since Wells (58) has shown that the absorbing force is proportional to the protein concentration of the blood, this decrease in absorption of water after hemorrhage was probably due to the fall of the blood plasma proteins. It is known, however, that distilled water is not a normal constituent of the small intestine and recently it has been shown by Dennis (12) that it is injurious to the epithelium of the ileum.

Anoxia and gastric impermeability. While anoxia may affect the permeability of the small intestine, no evidence has been presented to show that is affects the permeability of the stomach. A study has been reported (35) on the effect of severe degrees of anoxia on the permeability of gastric epithelium to water. The most severe degree of anoxia used in this study was a partial pressure of 53 mm. of Hg (28,000 ft.). Although animals were exposed to this degree of oxygen want for an hour or more, no appreciable influence on the absorption of water was found.

Absorption of sodium chloride. Hamburger (18) in 1896 reported that absorption of salt solutions in the intestine of dogs dead from one to twenty-four hours proceeded in the same manner as in living dogs. Magee and Macleod (23) in 1929 found that the walls of segments of gut became more permeable to solutions of sugars and electrolytes after devitalization.

Van Liere and Sleeth (51), working with barbitalized dogs and using the same technique as the senior author and his co-workers used with water, found that anoxia decreased somewhat the amount of absorption of physiological salt solution from the small intestine. Relatively mild degrees of anoxia (partial pressure of oxygen of 117 mm. Hg —8,000 ft.) caused a decrease in the amount of absorption. The absorption of fluid and the actual sodium chloride absorption ran parallel. The authors concluded from their work that oxygen aids in the absorption of physiological sodium chloride solution from the small intestine.

The author and his co-workers (49), using the same technique as just described, found that ephedrine administered either orally or intravenously had no effect on the intestinal absorption of isotonic sodium

chloride solution. In a subsequent paper (50) they reported that dogs which had been bled 3.2 per cent of their body weight absorbed more physiological salt solution from the small intestine than did control dogs. The results obtained with anemic anoxia, therefore, were different from those obtained with anoxic anoxia. The explanation offered was that after a severe hemorrhage there is a depletion of chlorides throughout the tissues of the body so that when the physiologic salt solution was placed in the intestine there was a higher diffusion gradient and the salt passed into the blood stream more rapidly.

Absorption of sodium chloride in the presence of a sulphate radical. It is known that the chloride ion is absorbed much more readily from the intestine in the presence of the sulphate radical (17). The mechanism of this is not understood. Van Liere and Vaughan (54), working with barbitalized dogs, studied the effect of various degrees of anoxia on the absorption from the small intestine of a solution consisting of equal parts of isotonic sodium chloride and isotonic sodium sulphate solutions. Anoxia depressed somewhat the absorption of the fluid and the sodium chloride; it did not, however, prevent the facilitation of the absorption of the chloride ion due to the presence of the sulphate radical. Anoxia had no effect on the absorption of sodium sulphate.

Absorption of the sulphate radical. Northup and Van Liere (28), working with barbitalized dogs and using degrees of anoxia ranging from a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) to a partial pressure of 53 mm. Hg (28,000 ft.), reported that the absorption of an isotonic solution of magnesium sulphate was not significantly affected. As previously mentioned, neither does anoxia have any appreciable influence on the absorption of sodium sulphate.

These studies indicate that even severe degrees of anoxia do not alter the permeability of the intestine to the sulphate radical. This is of some practical importance since magnesium sulphate in many instances is given to patients with cardiac disorders which are often associated with anoxic states.

Absorption of glucose. It has been shown by Gellhorn and Northup (15) that circulatory factors can affect glucose absorption but the effects are inconstant and therefore in a large series can be ignored. Northup and Van Liere (unpublished work) found that degrees of anoxia up to a partial pressure of oxygen of 63 mm. of Hg did not influence the absorption of isotonic glucose solution. When lower partial pressures of oxygen were used, however, glucose was absorbed more rapidly.

It has been shown (49) that ephedrine (which is known to cause

vasoconstriction of the splanchnic region) either given orally or intravenously did not influence glucose absorption. It also has been shown that anemic anoxia, produced by bleeding dogs 3.2 per cent of their body weight, had no effect on the absorption of isotonic solution from the small intestine. The fact that the absorption of glucose apparently involves a phosphorylization process rather than an oxidative one (Verzar and McDougal, 55) probably explains why no significant change is produced by moderate and even moderately severe degrees of anoxia. The increase of absorption of glucose produced by severe degrees of anoxia (partial pressure of oxygen of 53 mm. of Hg or lower), however, can best be explained by the increased permeability produced by the severe degree of anoxia.

Absorption of glucosides. Anoxia in ranges of partial pressures of 93 mm. Hg to 53 mm. Hg has been found to have no effect either on the absorption of digitalis *per se* or on the fluid menstruum in barbitalized cats (46).

Absorption of amino acids. Northup and Van Liere (unpublished work) found that the absorption of glycine was increased by moderately severe degrees of anoxia (partial pressures of oxygen from 80 to 63 mm. Hg) but at more severe degrees of anoxia absorption was markedly decreased.

Studies on the effect of anoxia on absorption show that the amount of interference with absorption is presumably of no practical significance and that ranges of anoxia compatible with life would not interfere, so far as absorption is concerned, with the proper nourishment of the body. Whether the increased permeability often brought about by severe degrees of anoxia would allow certain split-products of proteins to be absorbed which would cause allergic manifestations or other untoward effects remains to be proven.

The main value of these studies in absorption is the light they throw on the mechanism of absorptive processes and especially that of the rôle of physiological oxidations. The studies on glucose absorption has lent support, for example, to the theory that the mechanism of glucose absorption is a phosphorylization process rather than an oxidative one since its absorption is not decreased by anoxia. Similar studies with other substances have shown how anoxia may affect the permeability of the epithelium.

EFFECT OF ANEMIC ANOXIA ON GASTRIC SECRETION. In 1936 Alvarez and Vanzant (2) reported a study on a large number of human beings concerning the relation between the hemoglobin content of the blood

and free gastric acidity. They found that the mean gastric acidity fell off sharply and the incidence of achlorhydria rose rapidly when the hemoglobin fell below 12 grams corresponding to 72 per cent. From their work they felt there was evidence to indicate that loss of blood in both animals and man can temporarily lower gastric acidity.

Clinical studies in cases of idiopathic microcytic anemia were reported by Hartfall and Witts (19) in 1933. In a series of 137 cases, 80 per cent gave evidence of achlorhydria; these investigators did not feel, however, that the low acidity was secondary to the anemia. Goldhamer (16), working with patients suffering from pernicious anemia, found a direct relation between the erythrocyte count and the amount of gastric juice secreted in a given time.

Apperly and Cary (3) in 1936 showed experimentally that free acid disappeared from the stomach when the red cell count of the blood fell below a certain critical level.

It will be seen when anoxic anoxia is discussed that anemic anoxia has a more profound effect on depressing gastric juice than has the anoxic type. One reason for this may be the loss of chlorides during hemorrhage.

EFFECT OF ANOXIC ANOXIA ON SECRETION. *Effect on gastric secretion.* Bayeux (5) in 1911, working with dogs, showed that at an altitude of 14,000 feet the volume of gastric juice was diminished but that the titratable acidity was not affected. Delrue (11) in 1934 transported dogs from his laboratory to higher altitudes and reported that at an altitude of 8,000 feet the gastric juice showed a decrease in pH and in titratable acidity. Hellebrandt, Brogdon, and Hoopes (20) in 1935, using human beings and producing anoxia for short periods by use of a rebreathing apparatus, reported that anoxia of the precoma type caused no appreciable decrease in gastric acidity.

Sleeth and Van Liere (34) in 1936, working on the response of the stomach to water in barbitalized dogs, found that only after a partial pressure of oxygen of 53 mm. Hg (28,000 ft.) had been reached was there a definite diminution in the titratable acidity and in the chlorides. In 1939 Pickett and Van Liere (31) subjected Pavlov pouch and Heidenhain pouch dogs to various degrees of anoxia; the most severe degree used was a partial pressure of oxygen of 63 mm. Hg (24,000 ft.). They reported that anoxia caused a decrease in the volume of gastric juice in all the dogs and the more severe the degree of anoxia, the greater the decrease in gastric secretion. The pH of the gastric juice did not change in the Pavlov group until a partial pressure of 63 mm. Hg was reached,

but the Heidenhain group was affected at 80 mm. Hg (18,000 ft.). On the other hand, the titratable acidity in the Pavlov group was decreased at a partial pressure of oxygen of 80 mm. Hg and that of the Heidenhain group at a partial pressure of oxygen of 63 mm. Hg, that is, just reverse of the effects produced on the pH. Total chlorides in the Pavlov group were not influenced by the ranges of anoxia used; in the Heidenhain group, however, they were decreased at a partial pressure of oxygen of 94 mm. Hg (14,000 ft.). The authors concluded from their work that the Heidenhain group was affected by less severe degrees of anoxia than the Pavlov group.

Effect on intestinal secretion. Northup and Van Liere (39) in 1939 studied the effect of anoxia on intestinal secretion in barbitalized dogs. Intestinal secretion was stimulated by injecting a peptone extract prepared according to the method described by Nasset and Pierce (24). They concluded that the intestine had a low energy requirement for secretion since it was only when a partial pressure of oxygen of 53 mm. Hg (28,000 ft.) was reached that a slight depression of secretion occurred. This depression in secretion, moreover, was not statistically significant even though 15 animals were used.

These studies on the effect of anoxia on secretion apparently show that the gastro-intestinal tract has a low energy requirement for secretion, since it is capable of withstanding relatively severe grades of anoxia before it is significantly affected.

This brief resumé indicates the relative paucity of our knowledge concerning the effect of anoxia on the gastro-intestinal tract. While indeed a good deal more work is needed on the effect of oxygen want on the entire gastroenteric tract, the fields which appear particularly promising for fruitful research are those which have to do with the effect of anoxia on secretion and absorption.

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CARDIAC AUTOMATICITY AND RESPONSE TO BLOOD PRESSURE RAISING AGENTS DURING INHALATION ANESTHESIA

WALTER J. MEEK

Department of Physiology, University of Wisconsin Medical School, Madison

Ever since the discovery of anesthetic agents their action on the circulatory system has been a subject of great medical concern. In this review we shall deal with the inhalation anesthetics, chloroform, ether and cyclopropane, and their actions on the automatic properties of the heart. Particular attention will also be paid to the cardiac effects of the sympathomimetic amines administered during anesthesia.

By automaticity of the heart is meant the production of the inner stimulus, the spread of the excitation and the rhythm of the resulting contraction. While the heart is automatic in the sense that it continues beating for a time when separated from either its blood supply or the central nervous system, it is of course normally subject both to humoral and nervous influences. The anesthetics as well as the sympathomimetic amines may not only affect the heart directly but also indirectly by way of these humoral and nervous mechanisms. The more important of these are concerned with the activities of the cardiac medullary and mid-brain centers, reflexes originating in the carotid sinus and aortic arch and variations in the secretory activity of the adrenal medulla.

It would be possible to outline the review on the basis of those physiological properties of the circulating system with which we shall be concerned. Most workers in the field however think in terms of a given anesthetic, and for this reason each agent will be taken up separately. Of the known gaseous anesthetics chloroform has been the most thoroughly studied and a knowledge of its circulatory effects will also serve as a background for the entire group.

CHLOROFORM. The early work on chloroform had to do chiefly with the cause of the deaths which occasionally occurred during its administration. From clinical and physiological investigations a great diversity of opinion resulted as to whether the system most concerned

was the respiratory, cardiac or vasomotor. A great deal of this literature previous to 1900 has now mostly an historical interest. Of these early contributions only those need be cited which have pointed the way for later investigations. The older literature may be found in Heffter's Handbuch (82), Killian's Narkose (78) and in the standard pharmacologies.

Effect of chloroform on the heart rate. The action of chloroform on the rate of cardiac contraction is somewhat variable, depending on the way in which the experiments are carried out, the species of animal used, the dosage employed and the length of time the heart has been subjected to the anesthetic. It may be stated in general that no matter how the heart may be exposed to the agent the rate decreases with increasing concentrations and duration of the experiment.

Diastolic standstill has been described by Bandler (4) for the heart of Daphnia, by Pickering (114) for the chick embryo and by Carlson (28) and Meek (103) for Limulus. For the frog heart Dieballa (34) found that 0.126 per cent of chloroform in a saline solution produced cardiac standstill. Although weaker concentrations did not always slow the heart he never saw stimulation at any stage. Schmiedeberg (139) made similar observations on the frog heart as did Rasche (116). Kevdin and Trofimov (77) also produced cardiac standstill in the frog and found that $CaCl_2$ quickly restored activity. Of all the workers Hahn (63) is the only one apparently who reported acceleration of the frog heart with chloroform. She perfused the heart with 2.5 to 5 parts of chloroform per liter in calf's blood or serum.

For the turtle heart Vernon (164) observed that cardiac slowing is almost proportional to the chloroform concentration.

Excised mammalian hearts may always be finally arrested in diastole by sufficient chloroform in the perfusion fluid. However, for the dog heart isolated from the central nervous system by the method of Hering, Embley (45) noted no marked or abrupt changes in rate before the final stages of paralysis Sherrington and Sowton (150) stated that there was no alteration in frequency of rhythm incident to perfusion of the cat heart with low concentrations of chloroform in Ringer's solution. Chloroform in blood was much less effective than in saline or Locke's solution. Tunnicliffe and Rosenheim (161) reduced the rate of the heart by perfusing a quantity of fluid containing only 0.12 mgm. of chloroform. Using the Langendorff perfusion technique, Loeb (93) secured standstill in cat hearts with an 0.084 per cent chloroform solution. Winter (167) made similar observations. Rasche (116) ob-

served only a negative chronotropic effect when perfusing the mammalian heart.

With inhalation of chloroform several observers have described cardiac acceleration during the stage of excitement; Bock (20) for rabbits; MacWilliam (96) for cats and Cluzet and Petzetakis (31) for dogs. Halsey, Reynolds and Blackberg (64) also noted acceleration in dogs with doses so small that the reflexes still persisted. On the other hand L. Hill (73) has referred to cardiac slowing during the early stage of induction. According to Tiemann (154) light anesthesia may be borne by cats for an hour or more with no change in rate. For dogs on 2 per cent chloroform in air Embley (46) described a slow rate which might be prevented by vagotomy. Bradycardia was the sole change in rate described by Gaskell and Shore (54) for rabbits, by Hecht and Nobel (66) for dogs, rabbits and an ape and by Frommel (51) for guinea pigs. Beattie, Brow and Long (13) found that the normal rate of sinus discharge in cats dropped only slightly during the course of a 2 per cent chloroform anesthesia. With concentrations greater than 3 per cent the rate dropped steadily to as low as 120 per minute. In man the Glasgow Commission recorded slow pulses in 50 patients. Cluzet and Tixier (32) reported bradycardia in all 6 cases studied. I. G. W. Hill (72) found only three cases of bradycardia and one of acceleration in 15 clinical anesthesias but Steinfeldt (152) reported cardiac slowing in each of 8 patients studied.

The effect of chloroform on the heart rate in the early stages of anesthesia is then variable. The accelerations have doubtless been due to muscular movement and sensory stimuli of various kinds beyond the experimenter's control. The inhibitions of rate are also most likely examples of the well known vagal reflexes initiated by stimulation of the respiratory passages. Frommel (51) found that the bradycardia was reduced by vagotomy, atropinization or administration of the vapour by tracheotomy. Embley (46) also noticed the slowing was abolished by section of the vagi. In the dog and man these vagal reflexes are not easily induced, but Arthus (3) has described them in the rabbit when the nasal mucosa was stimulated by chloroform itself.

After anesthesia is established the reduced pulse rate persists or appears if there has first been acceleration. On this all workers agree. The slowing increases with the increasing depth of the narcosis until there is cardiac standstill. In these later stages the slowing cannot of course be reflex in origin and there is evidence both by Elfstrand (42) and Embley (46) that the vagi have lost much or all of their irritability.

Cardiac slowing in the later stages of the anesthesia must then be due to the direct effect of the chloroform on the heart or to indirect effects from the failing circulation.

Cardiac irregularities in chloroform anesthesia. Early observations both in the clinic and in the laboratory indicated that chloroform induced cardiac irregularities in addition to the changes in rate already mentioned. Actual stoppage of the heart which will be discussed in the next section was of course the most noticeable disturbance that could occur. Variations in conduction and rhythm were however evident though they could not be analyzed to advantage from the commonly noted pulse or blood pressure tracings. Advances were made possible by employing Engelmann's suspension methods and the venous pulse, but even then there could be no assurance of exactly what was happening until the development of electrocardiography.

Although Knoll (80) as early as 1878 had noted arrhythmia under chloroform and MacWilliam (96) in 1899 had described changes in irritability often in the form of a bigeminus, it was Levy and Lewis (92) who first showed by means of electrocardiograms that chloroform anesthesia may produce a series of irregularities from automatic centers in the ventricles ranging all the way from isolated extrasystoles to ventricular fibrillation. Hecht and Nobel (66) noted various disturbances in 15 dogs, 15 rabbits and 1 ape sometimes early but usually in the later stages of deep anesthesia. The P and T waves tended to be reduced in size and diphasic, the latter change being attributed to vagal influences. R was smaller, widened and notched. Auricular fibrillation was observed three times, once with an independent ventricular rhythm. Auricular extrasystoles were seen but once and ventricular twice. Only once did a group of ventricular extrasystoles lead into a tachycardia. There was but one spontaneous ventricular fibrillation although two appeared after massage in cardiac standstill. On the whole the irregularities, while present, were not as significant as those described by Levy and Lewis (92).

Nobel and Rothberger (108) were unable to demonstrate any arrhythmia in tracheotomized cats and dogs under light chloroform anesthesia (0.5–1 per cent), which seems at variance with the results of many other workers. In untracheotomized cats, however, irregularities appeared. In one animal subjected to 0.5 per cent chloroform scattered ventricular premature beats appeared. In another animal, first given 2 per cent vapour which was then lowered to 0.5 per cent, complete dissociation with a left sided ventricular autonomy was produced. In chloroformed

dogs Cluzet and Petzetakis (31) described lengthened P-R intervals and partial A-V blocks. Often there were long ventricular pauses broken by abnormal ventricular complexes. In prolonged anesthesia, negative P waves might appear and both P and T might become diphasic.

Frommel (51) working with guinea pigs found that the initial bradycardia of chloroform narcosis might pass into A-V block, complete but transitory. Instead of block there might be a salvo of extrasystoles. If the animals were allowed to succumb from the chloroform, the fatal termination might be a complete and permanent block or a decrease in both auricular and ventricular complexes passing finally into flutter and fibrillation. Following Levy's lead, Tiemann (154) studied cats in both light and deep chloroform anesthesia. In light anesthesia there were few irregularities unless preceded by a long period of deep narcosis. Deep anesthesia seemed to sensitize the heart to a succeeding lighter stage. In an anesthesia produced by chloroform of more than 1 per cent concentration, extrasystoles, nodal and particularly ventricular, were frequent. Light anesthesia alone was often endured for an hour or more with no change in rhythm. There were no changes in the P-R interval or in the shape of the regular waves of the electrocardiogram.

Eismayer and Wachsmuth (41) described an increase in respiratory arrhythmia at the beginning of narcosis which might lead to the development of automatic tertiary centers. A-V conduction disturbances were observed but the most significant change in the electrocardiogram was deformation of QRS complexes which was believed to indicate myocardial damage. Beattie, Brow and Long (12) state that with chloroform vapour of less than 2 per cent, cardiac irregularities do not appear even after 2 to 3 hours. With greater concentrations, however, the A-V conduction time lengthens and spontaneous abnormal QRS complexes occur, single, alternating with regular beats or in tachycardial groups. The T wave did not change unless the anesthesia was deep or the oxygen saturation of the blood seriously lowered.

Recently Chiarello (30) failed to observe arrhythmia in dogs during chloroform anesthesia. Oscillations in amplitude of the electrocardiographic waves, elevation of the S-T segment in leads I and II and inversion of T waves, were observed. The variations were believed to be due to transitory changes in the nutritive conditions of the heart and to be of no very great significance.

Studies of cardiac behavior under chloroform anesthesia have not been frequent in man. Cluzet and Tixier (32) in six clinical cases observed bradycardia and a slight reduction in amplitude of the waves of

the electrocardiogram. In only two of the six cases were there frequent extrasystoles. Hill (72) in fifteen cases found four unchanged under chloroform, three showed only minor variations, while the remaining eight exhibited marked disturbances, particularly extrasystoles of ventricular origin, one of which passed into a paroxysmal tachycardia. The disturbances generally disappeared as the anesthesia deepened. Six patients under chloroform were studied by Kurtz, Bennett and Shapiro (83). Four of these showed displaced pacemaker and two, extrasystoles.

Several attempts have been made to find the responsibility of the surgical procedures for the appearance of these irregularities. Although Rehn (118) thought the disturbances in clinical anesthesia were mostly nervous, due to reflexes from the operative field, Wachsmuth and Eismayer (165), Hill (72) and Maher, Crittenden and Shapiro (98) all agreed that the irregularities were a function of the anesthetic rather than of reflexes from the operative field.

From the above it is evident that cardiac irregularities are frequently observed in chloroform anesthesia, their occurrence depending chiefly on dosage, the species being studied and methods of administration. Most of the arrhythmias are reversible and disappear at once as the anesthesia is lightened and the animal regains consciousness. The literature so far cited refers to examples of smooth or what may be called normal chloroform anesthesia in which the administration of the anesthetic is skillfully carried out and all other conditions carefully controlled. In such cases though cardiac irregularities may occur, the anesthesia may be carried even to the paralytic stages of respiratory arrest while the heart continues to beat actively. At other times there are sudden circulatory collapses, the nature of which has given rise to a vast clinical and experimental literature.

Chloroform anesthesia and circulatory collapse. The problem of chloroform syncope came to the physiologists from the clinicians. Hardly had the agent been introduced before notices of sudden death under its influence began to appear. An interesting account of the early use of chloroform and ether in America has been gathered together by Hoff (74). In England the Registrar-General for 1890 reported thirty-six deaths from chloroform anesthesia. In 1899 (40) eighty-three deaths were reported and doubtless this was only part of the total.

The early studies of chloroform anesthesia had to do chiefly with the cause of these deaths. From clinical and physiological investiga-

tions a great diversity of opinion arose as to whether the system most concerned was the respiratory, vasomotor or cardiac. A great deal of fundamental work was developed in answer to this question.

Wood (168), MacWilliam (95), Gaskell and Shore (54, 55) and others clearly demonstrated that chloroform had a definitely poisonous effect on heart muscle and that death might be cardiac. But this by no means cleared up the mechanism of chloroform syncopeal death. Overdosage and myocardial paralysis did not seem to be determining factors, for in the clinics deaths were reported before the blood content of chloroform could apparently have become high enough to cause cardiac paralysis. The heart often stopped without gradually weakening, and if there was recovery, the beats were at once full and strong. Furthermore the deaths reported were usually in the early stages of anesthesia. During 1899 of the eighty-three deaths reported in England sixty-nine occurred before the operations were even started.

To explain the early deaths under chloroform anesthesia, Embley (45) (46) proposed vagal inhibition. In morphinized dogs by increasing the chloroform in the inspired air from 2 to 4 per cent, he found the beat was slowed or frequently suddenly arrested. These results were not prevented by section of reflex sensory paths from the respiratory organs but they were abolished by vagotomy or injection of atropine. Embley's idea was that chloroform raised the excitability of the vagus center, particularly in the early stages of the anesthesia, and that the inhibitory action was more intense because the poisoning of the heart muscle in some way rendered it more sensitive to the vagal stimulation. Embley's work was valuable in that it emphasized the importance of studying specific physiological mechanisms and in that it postulated an enhanced cardiac sensitivity. His use of morphine was undesirable. Although later workers found temporary stoppage of the heart in the early stages of chloroform anesthesia and that these might be removed by cutting the vagi (11), permanent inhibition could not be demonstrated. Probably some of Embley's inhibitions were really ventricular tachycardia or fibrillation.

The next attempt to explain cardiac syncope under chloroform was made by Levy (86, 87, 88, 89, 90, 91) whose ideas have markedly influenced all subsequent studies on the subject. In cats inhaling chloroform he noted that the blood pressure would often suddenly fall to zero and the animals die. On opening the chest the hearts were seen to be dilated and the ventricles in a state of fibrillation. At first he related these mishaps to the increased cardiac irritability resulting

from light anesthesia. Later (91) it became evident that the heart could be maintained beating at a perfectly regular rate even when lightly anesthetized, provided the anesthesia was level and unchanging, and no other disturbance was at work. The syncopes were conditioned by the change of cardiac state involved in the progress from deep to light anesthesia.

Working on the hypothesis that adrenalin might restore the circulation, Levy found on the contrary that doses as small as .03 mgm. were particularly efficacious in bringing on collapse. It is interesting that Oliver and Schaeffer (86) saw one case of collapse after injection of their adrenalin extracts into a chloroformed dog. Elliott (43) also described some accidents of the same nature but the anesthetic is not mentioned. Only MacWilliam (96) had previously noted any irregularities under chloroform.

In collaboration with Lewis (92), Levy investigated by means of the electrocardiograph the cardiac irregularities appearing under controlled concentrations of chloroform. In the cat after a few minutes' inhalation of 0.5 per cent chloroform ventricular extrasystoles regularly appeared. As the anesthetic increased to 1.5 and 2.0 per cent the irregularities disappeared but they returned as the anesthesia was lightened and often passed into a ventricular tachycardia. In deep anesthesia a dose of adrenalin as large as 0.065 mgm. induced only premature beats, but in light anesthesia as little as 0.016 mgm. caused ventricular tachycardia and fibrillation. Levy and Lewis believed that extrasystoles and ventricular tachycardia were stages on the way to ventricular fibrillation.

Independently Levy (86, 89) found that adrenalin produced fibrillation in light chloroform anesthesia after vagotomy, sympathectomy, pithing of the cord and in one case after the bulb was destroyed. It is notable that after pithing the cerebrum, 0.2 mgm. or nearly four times the usual dose of adrenalin caused only antecedent irregularities with momentary fibrillations. Stimulation of the stellate ganglia also precipitated fibrillation quite as well as the injection of adrenalin. Stimulation of the right accelerator mechanism was more effective than the left, later confirmed by Beritoff and Tschikamanauri (16), although Rothberger and Winterburg (130) had found that in the dog the left was more active in developing automatic ventricular centers. Under light anesthesia vagotomy was a good way to induce tachycardia which might proceed into fibrillation. This was easily explained on the assumption of an unrestrained sympathetic effect following the

loss of vagal tonus, but it was found that vagotomy was also effective following a previous section of the sympathetics which seemed to indicate that some intramuscular change of state was concerned. Heightened vagal tonus was not favorable for the development of fibrillation. Sensory stimulations of such nerves as the sciatic or splanchnics were followed by cardiac irregularities which occasionally led to ventricular fibrillation provided either the accelerator or adrenal nerves were intact.

Levy's ideas may be briefly stated as follows. Chloroform first renders the heart irritable, that is, liable to exhibit beats of heterogenic or ectopic origin. These actually occur only when the heart, sensitized by the chloroform, is subjected to some further exciting cause. Such simulations may be due to reflex increases of accelerator activity, reflex secretion of adrenalin, direct injection of adrenalin, or some change of state in the heart as exemplified by section of the vagi or varying the strength of the anesthetic, particularly from deep to light. Cardiac irritability is raised in light anesthesia and lowered in deep. Deep anesthesia, since the heart is depressed and not so irritable, is safer, but even light anesthesia if kept steady and not preceded by deeper stages (91) does not readily sensitize the heart. Sensitization is however readily induced by a change from deep to light anesthesia.

Levy did not believe in any over-dosage theory. His idea that cardiac irritability is greater under light chloroform has been borne out by the experiments of Tournade, Malmejac and Djourno (157) who showed that faradic stimulation produced fibrillation in light but not in deep anesthesia.

Shortly after Levy's series of papers, chloroform narcosis was again investigated by Nobel and Rothberger (108). Their findings in light chloroform anesthesia differed sharply from those of Levy in that they observed no irregularities, that injection of adrenalin with the exception of one fibrillation produced only extrasystolic rhythms, that section of the vagi decreased the effect of adrenalin and that sensory stimulation was ineffective in precipitating cardiac disturbances. These differences may have depended at least in part on different techniques, Nobel and Rothberger having introduced chloroform by way of tracheal cannulae into morphinized dogs. There was agreement on the general observation that adrenalin was more effective after light chloroform anesthesia than before.

While Nobel and Rothberger did not exactly oppose Levy's hypothesis that chloroform increases the irritability of the lower automatic

centers of the heart, they laid most emphasis on the vagus and accelerator mechanisms. Chloroform increased the activity of the cardioinhibitory mechanism as shown by Embley (45), Schaeffer and Scharlieb (135, 136) and a number of still earlier workers. Adrenalin also stimulated the vagus and after its injection cardiac standstill of more than 4 seconds was observed. If the dose of adrenalin was small, the marked sinus bradycardia allowed the escape of impulses, mostly from the A-V node. With larger doses of adrenalin sympathetic stimulation of the ventricular structures began to appear. These automatic centers, not being normally under any vagal control, responded first with a few scattered beats which might rapidly increase, developing into a multifocal ventricular tachycardia or even a ventricular fibrillation. Such a conception was borne out by the experiments of Rothberger and Winterberg (130) who brought on an A-V rhythm and occasionally fibrillation in the dog by simultaneous stimulation of the right vagus and left accelerator. Nobel and Rothberger's idea explained why cutting the vagi or administration of atropin rendered adrenalin less effective. Vagal stimulation was thereby eliminated, the sinus node was accelerated and to become dominant, ventricular automatic centers had to be stimulated to a much higher degree if ventricular tachycardia was to develop. van Dongen (37) has recently supported the same idea.

Both Levy and Nobel and Rothberger state that adrenalin injections in intact unnarcotized animals never led to cardiac irregularities, an observation out of harmony with the results of all recent workers. Nobel and Rothberger were the first who controlled all their experiments with electrocardiographic records. The observations of both these workers as well as most recent ones would be more significant and understandable if they had consistently given the weights of their animals, the doses of adrenalin in milligrams per kilo and the duration of all injections. Only on such a basis can comparisons really be made.

That ventricular fibrillation occurred in cats during the administration of chloroform as noted by MacWilliam and studied by Levy was confirmed by Embley (47) who observed it in about three of every five animals. He attributed it to an intermittent nervous control of the heart during the early anesthetic stages, marked reflex accelerator activity alternating with temporarily restored vagal control. In the dog an early chloroform syncope due to ventricular fibrillation was not observed by Embley (48) in several hundred experiments. If a collapse occurred it was a terminal process when blood pressure had fallen to a very low level. Syncope from chloroform in the dog seemed to be of

the vagal type only, being accompanied, however, by vasoconstrictor inhibition, vaso-dilator stimulation and myocardial depression. It was not easy to kill a dog with chloroform.

That there are definite species variations in susceptibility to chloroform and chloroform-adrenalin syncope is apparent from the literature so far cited. That chloroform did not strongly sensitize the dog heart to adrenalin was shown by Meek, Hathaway and Orth (105). Bardier and Stillmunkes (5) were unable to produce chloroform-adrenalin syncope in the frog, toad, eel, guinea pig or rabbit. Gautrelet and Halpern (56) also stated that the rabbit was refractory. It must be remembered however that some of these statements are apt to be misleading since they are based on only a limited study of dosages. It should be, but not always is, well recognized that the results depend in part on concentrations and duration of the injections. That dogs are much more resistant to chloroform than cats seems well established although Smirnow (151) inclined to the opposite view.

Bardier and Stillmunkes (8) produced chloroform-adrenalin syncope in dogs on doses of approximately 0.01 mgm. per kilo of adrenalin. It is to be noted that Meek, Hathaway and Orth (105) were unable to secure ventricular fibrillation with doses of adrenalin as small as this. Neither of these two groups of workers could demonstrate that the depth of the anesthesia was significant. Bardier and Stillmunkes agreed with Levy that the syncope was peripheral in origin and did not depend on the central nervous system. They believed that adrenalin served as a final stimulus to the heart already rendered irritable by chloroform, or that adrenalin suddenly reinforced the toxic action of the chloroform, an idea similar to that suggested by Richet. In a later publication (7) they strongly supported the latter view, though they presented no rigorous proof. In dogs they were unable to get ventricular fibrillation by stimulating the splanchnics, as Levy did in cats, and they found that there was actually a decrease in splanchnic irritability in chloroform anesthesia. For this reason they distinguished between chloroform syncope and chloroform-adrenalin syncope.

Smirnow (151) evidently produced chloroform-adrenalin fibrillation in dogs with ease but curiously not in cats under the same conditions. Tiemann (154) found that in cats cardiac irregularities were more common in deep than light chloroform anesthesia. He did confirm Levy in that ventricular fibrillation was most easily induced when the animals were carried from deep to light anesthesia. He found, however, under these conditions that fibrillation was not particularly frequent,

occurring in only four out of twenty experiments. Tiemann recognized two types of death from chloroform narcosis; one a diastolic standstill from overdosage and the other ventricular fibrillation.

Another interesting chapter on chloroform anesthesia was written by Beattie, Brow and Long (13, 23) who demonstrated that chloroform produced cardiac irregularities by virtue of its action on the hypothalamus. They found that in the cat anesthetized with 2 per cent or less of chloroform, extrasystoles occurred and could be maintained for long periods of time, provided the oxygen saturation of the blood and the respiratory exchange were kept constant. A Sherrington decerebration not only abolished any abnormal rhythm existing at the time but extrasystoles could not be produced later on inhalation of the optimum amount of chloroform vapour. It was therefore obvious that the region responsible for the arrhythmia was above the anterior colliculus. Its exact location was determined by appropriate transections and found to be in the hypothalamus, a few millimeters on each side of the mid line below the thalamic commissure. Direct stimulation of this region produced extrasystoles. Their production by stimulation of the hypothalamus was due first to stimulation of the general sympathetic pathway to the heart, as evidenced by their absence after extirpation of the stellate ganglia or section of the fibers supplying these ganglia, and second, to stimulation of the pathways controlling the secretion of adrenalin.

Under chloroform the stimulation of such nerves as the sciatic or radial called forth extrasystoles so long as either the sympathetic nerve connections to the heart or the adrenals were intact and the hypothalamus and thalamus were intact. Beattie, Brow and Long therefore concluded that the sensory side of the arc for the reflex secretion of adrenalin and for cardiac accelerator effects reached as high as the thalamus, from which direct connection was made to the hypothalamus. Although stimulation of the sciatic nerve did not call forth extrasystoles after midbrain transection it did cause a small rise in blood pressure. There were then synaptic centers below the hypothalamus which might still make the production of extrasystoles possible.

Many observations have been made on the relation of the cardiac nerves to chloroform and chloroform-adrenalin syncope, to some of which we have already referred. Nobel and Rothberger (108) got arrhythmia under chloroform only when the vagi were intact, but Beattie, Brow and Long (13) found that the spontaneous extrasystoles were not prevented by vagotomy. There is general agreement by

Levy (89), Bardier and Stillmunkes (5), Frommel (51), Heinekamp (67), Garrelon and Pascalis (52), Bouckaert and Heymans (21) and Bijlsma and van Dongen (19) that section of the vagi does not prevent chloroform-adrenalin fibrillation. Both Levy (89) and Bardier and Stillmunkes (5) have reported the chloroform-adrenalin response after the heart was deprived of all nervous connections, although it seems a larger dose of adrenalin was necessary.

According to Beattie, Brow and Long vagal stimulation abolished the extrasystoles from chloroform alone, and Levy (91), Embley (47) and Smirnow (151) have reported that vagal stimulation or increased vagal tonus as occurs with morphine, protected from the chloroform-adrenalin syncope. On the other hand Bardier and Stillmunkes (5) could not confirm such results, and Hill (72) and Parade (113) have reported the production of extrasystoles under chloroform in patients by mechanical stimulation of the vagus. Gautrelet and Halpern (56) have stated that vagomimetic drugs favor fibrillation.

That stimulation of the peripheral vagus might either cause or abolish cardiac irregularities has often been reported experimentally, but the explanations have always been disconcerting, especially in view of the generally accepted evidence that the vagus does not influence the ventricles directly. Scherf (137) on the basis of his studies with aconite, believed that the vagus freed chemical substances which increased the activity of ventricular centers. If one accepts the idea that irregularities may arise in different ways from the lower cardiac centers, some of the apparently contradictory results might possibly be explained. When single beats or even runs of tachycardia are regularly related or coupled to a normal one, they are not the simple expression of increased ventricular automatism but may be regarded as the rather passive response of a heterotopic center to a normal excitation wave which passes by. Such irregularities might easily be abolished or modified by vagal action. If the normal beats or rhythms originate independently from tertiary centers then the vagus would have no control over them. Since in the chloroform-adrenalin response there is a development of fibrillation through stages of extrasystoles and tachycardia, it might well be that the effect of vagus stimulation might vary, depending on the mechanism responsible at the moment for the irregularities. Goldberg and Rothberger (57) and Schott (140) have presented work in support of such ideas.

Section of the accelerators did not prevent a chloroform-adrenalin syncope in the experiments of either Levy (89) or Bardier and Still-

munkes (5), although the reviewer has found that the dosage of adrenalin must be significantly increased. In view of the work of Beattie, Brow and Long this probably means that the heart is being sensitized in part by impulses reaching it from the higher mid-brain centers. Accelerator stimulation has been shown to produce fibrillation by Levy (89), Bardier and Stillmunkes (5), Tiemann (154), and Beritoff and Tschikamanauri (16). In the cat the right accelerator is the more effective while the reverse is true for the dog.

Bouckaert and Heymans (21) have reported that denervation of the carotid sinuses and section of the aortic fibers protected from a chloroform-adrenalin syncope. Their explanation was that the unopposed sympathetic nervous action on the heart and the extra secretion of the adrenals prevented cardiac dilatation during the hypertension induced by the injection of adrenalin and so prevented fibrillation. This rests on the assumption that only dilated hearts fibrillate.

Most workers have held to the view that chloroform raised the irritability of the ventricular tertiary centers to a point at which some further exciting cause, particularly adrenalin, might readily induce extrasystoles, tachycardia or fibrillation. Instead of looking on adrenalin as a simple stimulant Bardier and Stillmunkes (7) regarded it as an agent which suddenly increased the toxicity of the chloroform. In support of this idea they quoted Delbert who found that adrenalin reinforced the action of chloroform. Garrelon and Pascalis (52) also believed that since adrenalin sensitized the organism to all anesthetics, the chloroform-adrenalin syncope was only a chloroform syncope in which the toxic action of the chloroform was reinforced indirectly by the adrenalin. The mechanism of reinforcement consisted of the addition to the circulation of a sensitizing thyroid substance demonstrated by Garrelon and Santenoise (53), the secretion of which was induced by the increased vagal tonus due to the adrenalin. In agreement with this hypothesis Garrelon and Pascalis found that a high section of the vagus did abolish the chloroform-adrenalin syncope while a low one was ineffective. A more rigorous proof would have been to test the dosage necessary to produce syncope before and after thyroidectomy. Hermann (70) has also accepted the idea that the toxic effect of chloroform is reinforced by adrenalin.

In the opinion of the reviewer the evidence that adrenalin strongly excites the automatic ventricular tissue of a heart already rendered highly irritable by chloroform, is too strong to be disregarded. Almost countless references might be cited showing the stimulating effect of

adrenalin on cardiac tissue independent of other factors. Reed and Smith (117) produced arrhythmia in the denervated frog heart; Bourne (22) showed that adrenalin was favorable to the production of ectopic beats in the dog's heart from electrical stimulation; Gorski (58) found not only that adrenalin stimulated the excised cat's heart but that it was thereby sensitized to further doses; Rothberger and Sachs (129) have quite recently shown the rhythmicity of auricular strips after treatment with adrenalin; and finally Nathanson (107) has induced cardiac standstill in the human heart and then produced automaticity in lower centers by an injection of adrenalin. Since ephedrine was not effective the result was attributed not to secondary effects but to direct adrenalin action.

There is also at present no evidence that adrenalin or that other sympathomimetic drugs act on the heart by way of mid-brain sympathetic stimulation. Marazzi (100) recently reported such an action on the preganglionic mechanisms but he was unable to demonstrate an increased outflow below the ganglionic depression. Pitts, Larrabee and Bronk (115) stated orally in their recent report that they had observed no adrenalin stimulation of hypothalamic outflow.

Although there are now a great number of sympathomimetic drugs, few but adrenalin seem to have been investigated in relation to chloroform anesthesia. Novadrenalin was found to be active by Tournade and Raymond-Hamet (159). Many other substances, however, are known to be able to bring on a chloroform fibrillation: nicotine (6), lobelin (160), hordenin (158), anagyren (156) and genet (27). All of these but genet may be assumed to act by increasing the secretion of the adrenals.

Many efforts have been made to find substances or procedures which would reduce or entirely prevent chloroform and chloroform-adrenalin syncopes. Gunn and Martin (62) recommended the intrapericardial injection of adrenalin itself for chloroform standstill in the rabbit's heart. More recently Tournade, Malmejac and Djourno (157) reported recovery in the dog from chloroform syncope by the intravenous or intracardiac injection of adrenalin combined with massage and artificial respiration. Henrijean (69) also cited the restoration of independent sinus and ventricular rhythms from injections of adrenalin. It is evident that these authors were dealing not with fibrillation but with a simple toxic depression of the heart, a real chloroform syncope. Although Tournade, Malmejac and Djourno found the treatment effective in most cases about one in ten dogs developed fibrillation that

was a true chloroform-adrenalin syncope. This danger had previously been pointed out be Bardier and Stillmunkes (7). Douglas (39) found that a preliminary injection of adrenalin some three minutes before the administration of chloroform protected from a later adrenalin injection, but this could not be confirmed by Tournade and Malmejac (155). The experimental literature in general would strongly recommend great caution in the use of adrenalin in chloroform anesthesia.

On the basis that chloroform-adrenalin fibrillation is due to the abrupt rise in blood pressure, as advocated by Shen (146), a number of substances and procedures which smooth out the blood pressure rise are reported to be protective; administration of hordenin (158), dioxane derivatives (146, 148) yohimbine (146), section of the presso-sensitive nerves (21), extirpation of the lumbar sympathetics (112), anagyrin (156), viper and scorpion venoms (8), restriction of the vaso-constrictor area (111), extirpation of the spleen and pancreas (111) and blockage of the reticulo-endothelial system (163). Protection has also been reported from the administration of morphine (151), $CaCl_2$ (77) (151), chloralose (121) and salt solution (120). Tiemann (154) found a loss of irritability to chloroform after parathyroidectomy, Bardier and Stillmunkes reported that adrenalin might be used as a restorative in chloroform anesthesia after hemorrhage and Hoff and Nahum (75) found that acetyl B methylcholin abolished chloroform-adrenalin irregularities.

Probably the most interesting of the long list are the agents which decrease the excitability of the heart itself and so eliminate the adrenalin effect. Such an action was found by Bardier and Stillmunkes for quinine (5) and quinidine (9) when given orally. Novocain has been shown by van Dongen (37) to protect the heart from electrically produced flutter and Shen and Simon (149) have used it to protect dogs from a chloroform-adrenalin syncope. According to Hermann and Jourdan (71) cocain injected intramuscularly increased the resistance of chloroformed dogs some three or four times to adrenalin. Dikshit (35) has reported that sodium barbital abolishes the irregularities of chloroform and adrenalin, without eliminating the blood pressure rise and they concluded that the irregularities were not due to pressor changes. The barbital effect is attributed by Knoeffel (79) to a diminution in adrenalin liberated by an over active sympathetic nervous system.

The idea that the chief difficulty in chloroform-adrenalin fibrillation might be the inability of the heart to withstand the intracardiac strain

imposed by the high blood pressure did not escape Levy. He examined the theory with care but felt forced to abandon it (90). Several later workers have, however, returned to the idea of intracardiac strain. Heinekamp (67) was impressed with the dilatation of a heart weakened by chloroform and its consequent inability to meet its load, particularly when driven by adrenalin. Beattie, Brow and Long (13) did not deny that the rise in blood pressure might have some effect in predisposing the heart to produce extrasystoles. They confirmed the observations of Levy that lowering the venous inflow to the heart abolished arrhythmia. Bardier and Stillmunkes (5) on the other hand found that a falling pressure from an acute hemorrhage did not prevent adrenalin from causing fibrillation. There was, however, a rise at the time of injection though it did not exceed the normal before hemorrhage. Evidence has been offered by Dikshit (35) that sodium barbital abolishes chloroform-adrenalin irregularities by its action on a mid-brain center and that the rise of blood pressure in itself is not directly effective on the heart. In his adrenalin studies on rabbits, Allen (1) has reported that if the adrenalin rise of blood pressure is prevented by a stabilizer, arrhythmia does not appear, but complete denervation of the heart does not prevent it. He believed that a rise of blood pressure from any cause will produce ectopic beats. Bouckaert and Heymans (21) attributed the stimulus for chloroform-adrenalin syncope to some kind of a sudden and intense change in the tonus of the vegetative nervous system.

Recently Shen (146, 147) has presented considerable evidence in favor of the importance of the rise of arterial pressure in determining the production of chloroform-adrenalin and benzol-adrenalin fibrillation. In particular the fibrillation fails to occur when the hypertensive action of adrenalin is either suppressed or reversed by previous injections of such substances as the dioxane derivatives or yohimbine. However, since van Dongen (38) has shown that the dioxane F933 makes the heart more resistant to electrical stimulation it may be that the protective factor is the direct restraining action on the heart and not the hypotension. For benzol-adrenalin fibrillation Shen concludes that the heart is intoxicated by benzol, predisposed to arrhythmia by the adrenalin and abruptly excited by the sudden rise in pressure. The same idea would doubtless hold for chloroform.

It is probably impossible at present to evaluate the exact part played by blood pressure rises on cardiac arrhythmia under chloroform although it is doubtless often a factor in the picture. It is well recognized

that an increased intracardiac pressure within limits stimulates the muscle fibers to greater contraction and it might well be that it can act as a stimulus to a heart rendered hyper-irritable by chloroform.

In the present state of our knowledge it seems impossible to arrive at any analysis of the chloroform effect on the heart which disposes it to fibrillation in terms of fundamental muscular physiology. The work of Sasaki (134) who gave evidence of a shortened refractory period of cardiac muscle under chloroform has been repeatedly quoted in this connection. MacWilliam (97), however, has reported no change in the absolutely, but a lengthening of the relatively refractory period. Van Dongen (37) found that novocain protected from extrasystoles and fibrillation produced in cats by adrenalin or $BaCl_2$ but there were no changes in the refractory period or conduction time. Bijlsma and von Dongen (19) have stated that strophanthin which forms fibrillation lengthens the refractory period, while ether, under which fibrillation seldom occurs, shortens the refractory period. These results give some support to the theory that fibrillation may be a heterotopic rhythm of great frequency and that drugs favoring fibrillation do so by stimulating the ventricular automatic mechanisms.

ETHER. From the circulatory standpoint ether resembles chloroform although the differences in action from a quantitative point of view are extremely great. Ever since their introduction as anesthetics, ether has been recognized as much less toxic than chloroform. A comparison of the two agents may be found in Beecher's *Physiology of anesthesia* (14).

The effect of ether on the heart rate and strength of contraction. Reviews of the older literature may be found in Heffter (82) and Killian (78). For hearts of the chick embryo (114), Limulus (28), fish (15), and frog (34) there is general agreement that in physiological saline ether has little effect in concentrations under about 5 to 10 parts per thousand but above this both rate and amplitude decrease until there is cardiac standstill in diastole. An initial stimulation has been observed by some workers.

In the perfused warm-blooded hearts stimulation from ether in the perfusing fluid has been reported rarely if at all. Bock (20) noted only slight depression in a rabbit heart-lung preparation. Tunnicliffe and Rosenheim (161) found that 0.2 per cent did not stop the excised heart. Loeb (93) produced diastolic standstill in the cat's heart with 1.7 per cent ether in blood-saline. Derouaux (33), Schramm (141) and Sarter (133) all reported depression in rate and amplitude as the

concentrations increased. Burridge (24) has stated that ether like chloroform has a twofold effect on the heart, an immediate depression followed by augmentation when the drug is removed.

That inhalation of ether always leads to a fast heart has long been recognized both in the laboratory and the clinic. In dogs (31) this occurs in the stage of excitation and remains during the entire period of narcosis (165). In guinea pigs it may be preceded by a period of slowing (51). Transitory changes in the early stages are doubtless due to reflexes from the irritating effects of the gas. Many workers have attributed the rapid heart rate to impairment of the vagal mechanism. Thus Kobacker, Lester and Rigler (81) found that the vagus was non-responsive to stimulation in cats when the animals were completely anesthetized. The site of ether action was at the peripheral ganglia since the pre-paralytic stimulation of nicotine disappeared but acetylcholine remained effective. Shafer, Underwood and Gaynor (145) noted that vagal stimulation affected the heart rate much less in etherized dogs than it did in the same animals after decerebration. Elliott (44), Cattell (29) and Bhatia and Burn (18) all believed that adrenalin was liberated during ether anesthesia. Samaan (132) has made the most careful study of the mechanisms responsible for the increased ether heart rate. By elimination at appropriate times of the vagi, cervical sympathetics, buffer nerves, splanchnics and adrenals he came to the conclusion that the increase in cardiac rate depended on 1, paresis of the vagal inhibitory mechanisms; 2, augmentation of cardio-sympathetic impulses; and 3, liberation of certain sympathicomimetic hormones such as adrenalin or sympathin.

Ether anesthesia and cardiac irregularities. The early workers were so impressed with the serious cardiac disturbances under chloroform that those observed under ether seemed minor and unimportant. Not until the electrocardiograph came into use were the cardiac irregularities in etherized animals carefully studied. The waves of the electrocardiogram for regular cycles initiated by the sinus are not particularly modified during ether anesthesia in laboratory animals. Small variations in the P waves have been reported by Cluzet and Petzetakis (31), Bettlach (17) and Chiarello (30). These possibly indicate slight displacements of the normal pacemaker. Others have noted the disappearance of the P wave and have interpreted it as nodal rhythm although Henrijean (69) thought this irregularity was not characteristic of ether anesthesia, the A-V node being less susceptible to ether than chloroform. Changes in the QRS complex described by Miller and

Felberbaum (106), Bettlach (17) and Foa (49) are limited to minor variations in voltage. Variations in the T wave have been noted by numerous investigators; Miller and Felberbaum (106) in cats and Eismayer and Wachsmuth (41), Bettlach (17), Chiarello (30) and Foa (49) in dogs. The T wave changes were usually reversals in direction, deep negative waves being often observed and believed by Hecht and Nobel (66) to indicate vagal action. In view of the variability of the T waves, particularly in the electrocardiogram of dogs, as shown by Barnes and Mann (10) and Katz, Soskin and Frisch (76), the changes described above probably have little significance. It should be noted that the modifications of the electrocardiographic waves under ether are reversible and usually quickly disappear when the anesthesia is terminated. Routier (131) regarded them as variations within physiological limits.

Blocks and ectopic beats are rare in light ether anesthesia, but as narcosis deepens they may begin to appear. Striking as they may be, they rapidly disappear on removal of the ether. There is no tachycardia from secondary or tertiary centers. The absence of rapid ventricular rhythms is in sharp contrast to what occurs under chloroform. Details may be found in many of the references already given, but particularly in the papers of Frommel (51) and Miller and Felberbaum (106). The latter authors reported that in twelve deeply etherized dogs partial and complete A-V block occurred three times each, nodal rhythm three times, ventricular extrasystole once and auricular fibrillation and flutter once each. Brow, Long and Beattie (23) mentioned that ventricular extrasystoles are found under ether but not as many as under chloroform. Even if the ether was crowded to cardiac standstill Henrijean (69) observed that the EKGs of the last beats were of the normal type. Frommel (51) and Eismayer and Wachsmuth (41), however, found that as the respiratory pauses lengthened and the heart began to fail extrasystoles from secondary or tertiary centers appeared and finally passed into ventricular rhythms with bizarre QRS complexes. Such results are in agreement with the experience of most laboratory workers.

Studies of cardiac irregularities in clinical anesthesia with ether are not too numerous. The results on the whole agree with those already described for laboratory animals. Only Cluzet and Tixier (32) and Unger and May (162) found no modifications of the EKG or irregularities under ether. Heard and Straus (65) had one nodal rhythm in twenty-one cases, a rather low incidence for this type of abnormality. Levine (85) in eight cases observed auricular tachycardia twice and

auricular fibrillation once. Lennox, Graves and Levine (84), Marvin and Pastor (101) and Steinfeldt (152), in one-third to one-half of their cases have all described irregularities consisting of displacements of the cardiac pacemaker, auricular tachycardia, nodal rhythm and premature auricular and ventricular beats. Wachsmuth and Eismayer (165) noted similar irregularities which they attributed to the anesthetic and not to sensory impulses from the field of the operation as Rehn (118) had proposed. Hill (72) associated the irregularities with the stage of induction. He noted displacements of the pacemaker, variations in P-R intervals but only rarely extrasystoles or extrasystolic rhythms. Maher, Crittenden and Shapiro (98) observed nodal rhythm in thirty-two and QRS changes in fifteen of seventy ether cases. Deep anesthesia was the most important factor associated with these changes. They could not be related to surgical procedures and they all disappeared when the anesthesia was discontinued. Kurtz, Bennett and Shapiro (83) included twenty cases of ether anesthesia in their recent studies. Over 65 per cent of these showed downward displacements of the pacemaker while in only 20 per cent were extrasystoles observed. Extrasystoles were more frequent in patients with heart disease than in those with normal hearts. It is noteworthy that the extrasystoles arose in all cases from the auricle or A-V node and never from the ventricle.

From both the laboratory and the clinic it may be safely concluded that ether does somewhat favor certain forms of cardiac arrhythmia. These are mostly delays in A-V conduction, partial blocks, A-V rhythm and premature beats. Ventricular tachycardia and ventricular fibrillation are rarely if ever observed. For the most part the sinus pacemaker maintains its dominance as pointed out by Schlomovitz (138). The irregularities are most apt to occur early in induction or near the level of respiratory arrest, though they are to be observed in all stages. They are evidently not particularly related to operative procedures. The effects of increased CO_2 and O_2 deficiency have not been thoroughly studied. All the disturbances disappear on discontinuing the anesthetic.

Ether and adrenalin. Levy (90) was not able to produce fibrillation in cats under ether by injecting adrenalin, an observation confirmed later by Bardier and Stillmunkes (5) for dogs. The statement of Papilian, Cosma and Russu (110) that an adrenalin syncope cannot be produced in animals under ether is, however, too far reaching, for the results depend upon the dose of adrenalin administered. Nobel and Rothberger (108) secured a fast left sided ventricular tachycardia in the cat. Fibrillation may be produced in any animal, normal or anesthetized, if adrenalin is given in large enough quantities.

The exact amount of adrenalin or others of the sympathomimetic amines necessary to produce ventricular tachycardia or fibrillation in animals under ether has not been determined, a matter well worthy of investigation. That the definite dose is larger than for chloroform or cyclopropane has been clearly shown by Meek, Hathaway and Orth (105). This is not evidence, however, that ether has decreased the normal sensitivity of the automatic tissues to the drug. It may even have increased it but nothing like to the extent of what is found for chloroform or cyclopropane. Van Dongen (36) has recently reported that ether favors the fibrillation produced by electrical stimulation but that it has no effect on the development of the rhythms caused by $BaCl_2$ or adrenalin. Ether thus facilitated the formation of stimuli by electrical currents but had no effect on the formation of stimuli by chemical agents.

An ether-adrenalin syncope comparable to that of adrenalin and chloroform is thus not found either in the laboratory or the clinic. Fibrillation may obviously occur in ether anesthesia from other causes but any relation to the anesthetic has not yet been demonstrated.

CYCLOPROPANE. The most recent inhalant to attain widespread popularity has been cyclopropane. Though first prepared by A. von Freund in 1882 (50) its anesthetic properties were not investigated until 1929 by Lucas and Henderson (94, 68). Waters and Schmidt (166) and Stiles, Neff, Rovenstine and Waters (153) introduced cyclopropane as a clinical anesthetic in 1934. The literature on all aspects of the new anesthetic is well covered in a recent monograph by Robbins (126).

The effect of cyclopropane on heart rate and strength of contraction. Waters and his associates (166, 142) stated in their early work that cyclopropane decreased the heart rate both in men and dogs. That the rate in dogs actually increases was shown by Meek, Hathaway and Orth (105) who recorded an increase in fourteen out of seventeen trained animals. Robbins and Baxter (123, 124, 125) have also found a greater heart rate in non-premedicated, untrained and trained dogs under cyclopropane anesthesia. Although the resting rate of the trained dogs was lower than the untrained the final rate in stage III_{2-3} was the same for both groups, an average of 146 to 149 per minute. In man the heart rate is usually reduced below the resting level (143). Shackell and Blumenthal (144) have noted that Rhesus monkeys react like man and that the higher the concentration of cyclopropane the slower the rate.

The exact mechanism by which cyclopropane increases the heart

rate in dogs has not yet been carefully analyzed in the quantitative way that Samaan (132) has used for ether. That the cardio-inhibitory center is most concerned is evident, however, from work by Robbins, Fitzhugh and Baxter (128). They noted that removal of the sympathetic innervation of the heart in dogs had little effect on the resting rate. Furthermore this bilateral removal of the sympathetics under cyclopropane did not immediately alter the heart rate, as it should if the rate were being maintained by accelerator stimulation (128). There is no record of cutting the cardiac vagal fibers under cyclopropane to find the exact extent to which the anesthetic had decreased vagal tone. There can, however, be little doubt that this is the method of acceleration.

After premedication with morphine, however, Robbins and Baxter (125) noted that cyclopropane further decreased the already slow heart rate. In thirteen morphinized dogs the average rate was 73, which was reduced to 68 under cyclopropane anesthesia. Wherever the anatomical point of action of morphine may be, there has never been any question that it was on the vagal mechanism, and that the net result was an increase of inhibitory action on the normal pacemaker. If the action of cyclopropane is reversed after morphine it would seem to be an example of chemical rather than physiological synergism.

Cardiac irregularities under cyclopropane. It is common experience that non-premedicated animals may generally be anesthetized with cyclopropane and carried to the point of intercostal paralysis, stage III_3, without signs of cardiac arrhythmia or abnormal changes in the electrocardiogram. Seevers, Meek, Rovenstine and Stiles (142) reported that only three dogs out of twenty-seven showed extrasystoles before stage III_3 and recently Robbins and Baxter (125) found only one in forty. Up to this stage changes in the electrocardiogram are negligible. Bettlach (17) noted that negative T waves became positive in leads II and III but remained negative in lead I, while they were markedly deepened in lead IV. The records presented by Robbins have shown no details of interest.

Although as just stated an animal may be carried indefinitely in a moderate degree of anesthesia under cyclopropane without any signs of cardiac disturbances, that irregularities do occur under certain conditions is equally well known. As Beecher has stated, the ultimate value of cyclopropane may well depend on the evaluation of its cardiac effects.

In their first pharmacological studies of cyclopropane Lucas and Henderson (94) noticed irregularities on their blood pressure tracings

which appeared to be missed beats. These were more or less temporary and disappeared as the concentration of the gas was diminished. Waters also observed during preliminary clinical experiments that an occasional patient developed some cardiac arrhythmia if the concentration of the anesthetic was high. This led to a study of the respiratory and electrocardiographic changes in cyclopropane anesthesia by Seevers, Meek, Rovenstine and Stiles (142) in which it was shown that the irregularities which had been observed were not due to any contaminants such as propylene, but to the gas itself. These authors correlated the alveolar concentration of cyclopropane with the stages and planes of anesthesia first described by Guedel (60), respiratory rate and volume and cardiac rhythm. Surgical anesthesia in dogs was secured with a mixture of 33 per cent of cyclopropane in oxygen. Respiratory arrest appeared at 39 per cent. Premedication with morphine reduced these percentages. Later analyses by Robbins (122) on both mixture inspired and blood showed a remarkably close agreement with these findings.

Cardiac irregularities according to Seevers et al began to manifest themselves at about the time respiration ceased, although there was considerable variation and seven out of twenty-seven animals showed extrasystoles before respiratory paralysis. The types of arrhythmia were auriculo-ventricular block, nodal and ventricular extrasystoles, nodal and ventricular rhythms, ventricular tachycardia and auricular and ventricular fibrillation. With the exception of the two ventricular fibrillations the heart returned in all cases to its normal rhythm as the anesthesia was lightened or artificial respiration instituted.

Seevers and his collaborators noted that irregularities were abolished by atropine but that they reappeared on increasing the concentration of the anesthetic. They believed this indicated a vagal origin for the disturbances although it is more likely such an effect is only vagal indirectly since with the increased heart rate under atropine the sinus rate is so high the lower automatic centers cannot express themselves. These authors clearly recognized that oxygen lack was a factor in producing the irregularities as they disappeared on the addition of oxygen or institution of artificial respiration. However, since they reappeared at higher concentrations of cyclopropane under artificial respiration and since they observed two deaths by fibrillation at a time when alveolar oxygen was sufficiently high, they emphasized the toxicity of cyclopropane itself.

Robbins and Baxter (123) have confirmed the work of Seevers et al.

by making in addition analyses of the blood for oxygen, carbon dioxide and cyclopropane. In eleven animals they found that the concentration of cyclopropane in the arterial blood at the time of respiratory arrest was 33 mgm. per 100 cc. and that on the average 6.0 minutes later cardiac irregularities began to appear. Seven of the animals recovered on the institution of artificial respiration, and irregularities under this condition did not appear the second time until the concentration of cyclopropane in the blood reached 43.6 mgm. per 100 cc. Section of the vagi had little effect on these results. Arterial oxygen of course rapidly declined after respiratory arrest and at the time cardiac irregularities appeared it averaged only 2.8 volumes per cent.

Robbins and Baxter were quite naturally impressed with the low oxygen values present at the time arrhythmia appeared and knowing that Greene and Gilbert (59), Mathison (102) and Resnick (119) had demonstrated similar irregularities in oxygen want, they concluded that the cardiac irregularities developing during the period of respiratory arrest were not due to any effect of cyclopropane on the heart but to the extreme degree of anoxemia. It is the opinion of the reviewer that this conclusion is too sweeping since it has been observed by Robbins and Baxter, Seevers et al. and others that in both man and animals irregularities do occur occasionally or even frequently before there is any significant degree of anoxemia. That cyclopropane has affected the heart long before the stage of respiratory arrest is also clearly shown by the work of Meek, Hathaway and Orth (105) to be described shortly. Robbins agrees with Seevers et al. that in higher concentrations cyclopropane does have a direct effect. It would be curious if it had no effect in the lower dosages. However, that a decrease in the oxygen content of arterial blood accentuates the cardiac irregularities and may be the chief factor in bringing them on is a point well proven by Robbins and Baxter and it is of great practical importance.

That morphine administered before cyclopropane predisposes the hearts of dogs to irregularities such as A-V block, nodal rhythm and ventricular extrasystoles has been observed by Robbins, Fitzhugh and Baxter (128). It should be noted that the cases studied by Kurtz, Bennett and Shapiro (83) in which extrasystoles and multifocal ventricular tachycardia appeared, had all received morphine and scopolamine or atropine. Although the dose of morphine used clinically may not be large enough to be dangerous in this connection, its action under cyclopropane should be more carefully studied.

Both sodium barbital and amytal have been found by Robbins and Baxter (127, 125) to protect against the cardiac irregularities induced

by cyclopropane. Cardiac arrest occurred much later after respiratory arrest than in the controls; only two of fourteen dogs receiving the barbiturates showed irregularities; and the arterial content of oxygen was higher at the time of respiratory arrest. These results justify the author's suggestion that such premedication be tried in man.

Guedel (61) has recently reported protection from cyclopropane arrhythmias by evipal, pentothal and nembutal. Of these pentothal was about 90 per cent efficient but evipal seemed more desirable since it was 80 per cent efficient and longer lasting. Guedel believes that there is a definite arrhythmic range in cyclopropane anesthesia beginning at about the point of respiratory standstill and continuing until the concentration of the gas reaches 40 per cent or more. If the concentration of the cyclopropane is further increased by artificial respiration the irregularities disappear, but they reappear and are more marked on the ascent from the deep anesthesia.

Effect of cyclopropane on the irritability of the automatic tissue. It is not enough in judging an anesthetic merely to note that the heart shows no arrhythmia. The normal pacemaker might be approaching a stage of inhibition which would allow escape phenomena, or ectopic centers might be on the point of exhibiting activity, should an additional stimulus appear. This reasoning led Meek, Hathaway and Orth (105) to test the condition of the automatic tissue of the hearts in controlled stages of cyclopropane anesthesia by a standard injection of adrenalin. A dose of 0.01 mgm. per kilo in 5 cc. of normal saline was chosen and this was injected intravenously at the rate of 1 cc. per 10 seconds. While the standard injection of adrenalin in seventeen unanesthetized controls produced the usual number of extrasystoles which were interpreted as escape phenomena due to the slow sinus rate, there was only one example of ventricular tachycardia and ventricular fibrillation never appeared. Under light cyclopropane anesthesia adrenalin produced ventricular tachycardia in eleven of the seventeen animals and one succumbed to fibrillation. Under deep anesthesia sixteen of seventeen dogs showed ventricular tachycardia and one died of fibrillation. The tachycardias were multifocal in type. In light anesthesia their duration was 19 seconds and this was increased to 44.5 seconds in deep anesthesia. The effect was directly related to the depth of the anesthesia. These conclusions were confirmed in a later paper by Orth, Leigh, Mellish and Stutzman (109) who found in twenty animals that all showed ventricular tachycardia with adrenalin and cyclopropane and five succumbed to fibrillation.

Similar experiments with many of the same animals under chloroform

and ether demonstrated that in dogs cyclopropane had a more marked stimulating or sensitizing effect on the ventricular automatic tissue than either of the other agents.

Although these experiments showed beyond question that adrenalin was contraindicated in cyclopropane anesthesia they should not be taken to mean that cyclopropane is a particularly dangerous anesthetic. The irregularities with the exception of fibrillation are easily reversible and cyclopropane has the advantage over most anesthetics in that the tissues may be quickly desaturated. Danger may thus usually be quickly averted.

Since adrenalin injected during cyclopropane anesthesia resulted in serious cardiac irregularities, Orth, Leigh, Mellish and Stutzman (109) investigated the action of other blood pressure raising amines. A series of twenty dogs were injected with the standard dose of adrenalin for controls. All of these showed ventricular tachycardia and five ventricular fibrillation. By using doses approximately equivalent to 0.01 mgm. of adrenalin in blood pressure raising power they found that the following amines in cyclopropane anesthesia acted on the ventricular automatic tissue similarly to adrenalin; arternol, epinine, kephrine and cobefrine. By the same methods it was found that ephedrine, propadrine, benzedrine, paredrin, synephrine and neosynephrine did not exert any such cardiac effects. With the exception of noeosynephrine they did however markedly accelerate the sinoauricular rate. In the dog under cyclopropane, neosynephrine is the sympathomimetic amine most favorable to the heart.

There is a wide variety of side chain groupings in the above mentioned drugs. Adrenalin has a side chain of methylamine ethanol; arteronol has amino ethanol; epinine, methylamine ethane; kephrine, methylamino keto ethane; and cobefrine, amino propanol. It seems however that these side chains must always be attached to a 3,4 dihydroxy ring in order to be very effective. Agents with only one or no hydroxyl did not stimulate the heart sensitized by cyclopropane. The strong stimulating action of the amines with two hydroxyls was not seen under all anesthetics. In ether anesthesia neither ephedrine, arterenol, cobefrine or neosynephrine produced ventricular tachycardia. Under chloroform some cardiac sensitization was shown to cobefrine and arterenol, less to ephedrine and none to neosynephrine.

Protection from the stimulating effects of sympathomimetic amines under cyclopropane. It was shown by Meek and Seevers (104) that after premedication with sodium barbital there is a higher degree of

protection against the cardiac effect of at least one of the sympathomimetic drugs, ephedrine. This suggested to Orth et al. (109) that the cardiac action of blood pressure raising agents might be reduced under cyclopropane provided the animals were previously barbitalized. Intravenous injections of 200 to 225 mgm. of sodium barbital per kilogram, 50 mgm. of amytal per kilogram and 25 mgm. of nembutal were therefore made before induction. When the anesthetized animals were fully under the barbiturate, the test dose of adrenalin was given. No evidence of protection could be secured, both ventricular tachycardia and fibrillation frequently appearing. These results are not necessarily in contradiction to the protection from cyclopropane effects alone reported for barbiturates by Robbins and Baxter (125) since the heart was subjected to two sets of stimulating influences instead of one.

Recently Burstein and Marangoni (25) reported that 5 mgm. per kilo of procaine given either before adrenalin injections or during the stage of ventricular tachycardia reduced the incidence of ventricular fibrillation in dogs during cyclopropane anesthesia. These results have been confirmed by the reviewer. Since the injection of procaine solution into the circulation of man is frequently followed by untoward reactions, Burstein, Marangoni, De Graff and Rovenstine (26) (99) have recently investigated the protective action of less toxic substances of the same chemical group, para-amino benzoic acid, paramon and sodium para-amino benzoate. Administration of these drugs prior to a test dose of adrenalin during cyclopropane anesthesia reduced the incidence of ventricular fibrillation. These authors also found that the intracardiac injection of procaine at the time of fibrillation in dogs effected a return to normal in a number of cases. The other p-amino benzoic acid derivatives were not effective. A large number of pharmacological agents remain to be studied in this connection.

Allen, Stutzman and Meek (2) have recently shown that after decerebration in dogs under cyclopropane anesthesia the test dose of adrenalin no longer produced ventricular tachycardia. The integrity of some center above the pons was thus necessary for a cyclopropane-adrenalin response. Lesions of the pons at the level of the fifth nerve, removal of the stellates with all connections from the upper fifth thoracic ganglia and the intravenous injection of ergotamin also abolished the response. The authors concluded that cyclopropane sensitization of the heart took place because the anesthetic stimulated a mid-brain center which then sent impulses to the heart by sympathetic pathways. The direct action of adrenalin on the heart thus sensitized produced the ventricular

tachycardia. The mechanism of action for cyclopropane was thus shown to be similar to that described by Beattie, Brow and Long (13) for chloroform. No evidence for mid-brain sympathetic stimulation of adrenalin could be found by Allen, Stutzman and Meek.

In decerebrated dogs under cyclopropane anesthesia abrupt rises in blood pressure may still be produced by the sympathetic amines without the appearance of ventricular tachycardia or fibrillation according to Allen, Stutzman and Meek. These results are at variance with the ideas of Shen et al. (148) who have postulated the height or abruptness of the rise in blood pressure as the cause of chloroform-adrenalin syncope.

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PHYSIOLOGY OF ITCHING

STEPHEN ROTHMAN

Section of Dermatology of the Department of Medicine and Department of Pharmacology, University of Chicago

"Itching is an unpleasant cutaneous sensation which provokes the desire to scratch." This trivial definition, about 200 years old (32), has not been modified in spite of advancing knowledge. It has been used to differentiate itching from other sensations which at times have been compared or confused with itching although they do not provoke the desire to scratch. Thus the sensation of vibration which is a function of the touch sense and is elicited by touch stimuli of high frequency has nothing in common with itching (22). Neither has the "formication" or "prickling" sensation which is perceived in circulatory arrest of the limbs, and which, according to recent research, arises from deep seated receptor organs under the skin (20).

"Superficial tickling" however seems to correspond with the above definition. If a light hair is drawn across the skin or neighboring mucous membranes, the sensation will be called a tickle, nevertheless in most persons it leads to the motor response of rubbing or scratching (55). For many years superficial tickling was regarded as a sensation different from itching (38, 71, 24), particularly after von Frey advanced experimental data supporting the assumption that tickling is elicited by stimulation of tactile receptors if the touch stimulus is weak, small and wandering, whereas itching is a function of the pain sense (24, 25). However, von Frey himself pointed out that by moderately increasing the intensity of such a stimulus, by increasing the speed of its movement and by repeating it on the same spot the sensation assumes a quality of itching. In his last review on cutaneous sensations von Frey (27) admitted that tickling might be a mixture of pure tactile impulses and itching. He also pointed out the great difference between a weak tactile sensation and tickling, the first being "objectivated", that is, related to external objects, and the second being "somatized", that is, related to the body or felt as an excitement of the body itself. It will be shown in this review that tickling and itching are identical. Electrophysiological analysis has revealed that both represent impulses of the

same nerve fibers and central tracts. Clinical experience conforms with this statement [see also (23, 26)].

Experimental itch stimuli. The first experiments on itching were carried out with "itch powder" (powdered filaments of the plant Mucuna pruriens, used by children in practical jokes). After a short period of pricking pain and a sensationless interval, it provokes pure itching in its full psychic content (57, 36). With itch powder a rather large surface of the skin was always stimulated, and it was assumed that a synchronous stimulation of numerous nerve endings is necessary to produce itching. In fact, it was found (25) that itching arises more easily from any suitable stimulus, especially from weak pain stimuli, if numerous neighboring skin points are stimulated. By such multiple stimulation a mutual accentuation of the impulses occurs. High frequency of repeated stimuli at the same place also furthers the inducement of itching. On this basis swinging tuning-forks (77) and faradic stimulation (16) were used to evoke itching experimentally. However, neither synchronous nor successive multiplicity of the stimulus is absolutely necessary to cause itching. It can be provoked by single, point-like, weak pain stimuli (4, 25). At present histamine punctures are preferred as experimental itch stimuli (7).

The whole body surface responds to itch stimuli. It is remarkable, however, that the highest pruritic sensitivity is found around the large openings of the surface in the transitional tissues between skin and mucous membranes (51).

Itching arises exclusively in the epidermis or in the corresponding epithelial layer of the transitional mucous membranes. It is impossible to elicit itching from skin areas denuded of their epidermis (71, 36).

Physiologic pruritus. Physiologic pruritus is a term used to contrast the pruritus of pathologic processes with the itching which arises in response to weak stimuli of every day life, such as slight rubbing, slight changes in pressure, and temperature (e.g., in undressing). The impulses resulting from such stimuli are of minimal intensity and scarcely enter the consciousness. However, a person may become conscious of this "minimal pruritus" when his attention is concentrated on it as occurs in forced immobility, in boredom and in fatigue (61). The instinctive desire of animals for cleanliness is supposed to be caused by it (35).

Itching evoked by pathologic processes in the skin or nervous system is merely an intensification of the physiologic pruritus and no sharp limit can be drawn between the two. However, such a distinction is

practicable if pathologic pruritus is interpreted as itching evoked by morbid stimuli or of such intensity that it causes disturbances of well-being. The most important feature of pathologic pruritus is the vicious circle of itching and scratching (see p. 376) which does not occur in physiologic pruritus although the latter is followed by short periods of scratching.

Itching and pain. Since the first experiments on itching were made, it has been obvious that the itching sensation is somehow related to cutaneous pain. It was found that in pathological cases of sensory dissociation itching could not be elicited in analgesic areas with intact touch sense, as in leprosy, syringomyelia and other lesions of the spinal cord (71, 4), and, conversely, itch sensation could be obtained in skin areas with complete tactile anesthesia and unimpaired pain sense, as in artificially induced sensory dissociation and in tabes (77, 57). Experiments on the normal mucous membranes showed the independence of itching from the temperature senses (57).

Special points on the skin surface which would respond with itching alone to any stimulus, such as is the case with touch, pain, warmth, and cold points, could not be detected but it was found that pain and itch points have the same density and that they cover each other (25). The interpretation of all these results was that itching is mediated by pain receptors and pain fibers. Some contradictory data concerning the relations of itching to tactile receptors apparently were based on too broad a definition of itching (16, 17, 40).

Further observations indicated that itching might be elicited in *hypoalgesia* by stimuli which normally caused pain. If the sensory functions of the skin were paralyzed by local anesthetics, locally or intraspinally, responses to pain and itch stimuli disappeared and reappeared at about the same time and independently of touch sensitivity. However, during the periods of increasing and decreasing anesthesia there were phases of hypoalgesia in which pain stimuli did not elicit pain, but itching (69). Similar observations could be made if the successive breakdown and recovery of skin sensations were provoked by blocking and releasing the blood circulation in limbs (49). In skin areas with *hypoalgesia* due to lesions of the spinal cord, slight pain and itching could hardly be differentiated. In such cases slight pin pricks were not felt at all; stronger pricks elicited an unpleasant sensation, called "itching pain" by the patient (57).

However, itching can be observed also in *hyperalgesic areas* and here elicited more easily than normally. Goldscheider (30) found that if

the skin is crushed in a small spot, a large area around the injury becomes *hyperalgesic* and in this zone itching can be elicited by subthreshold pain stimuli. Itching arises in such areas also by "inadequate" stimuli such as light point-like touch, cold and warmth.

The main difficulty in attributing itch sensation to impulses of pain fibers was seen in the fact that subjectively pain and itching are different sensations and call forth different motor responses. It seemed that an identification of pain and itching is incompatible with Muller's law of the specific energies of sense organs. This law, however, "has hitherto resisted all attacks, and all new facts in the physiology of sense organs have satisfied it" (79).

To overcome this difficulty, reference was made to the obvious unhomogeneity of the cutaneous pain sensation itself (pricking, stinging, burning, throbbing pain, etc.) and attempts were made to find a pain quality which in its psychic content is more closely related to itching than is the total complex of pain sensations; thus, the view was advanced that itching arises from impulses of the protopathic fibers of Head, itching being nothing else but a weak protopathic pain (57, 59).

Criticism of Head's dualistic theory. Head's theory (33, 34) was based on his findings in skin areas after the sensory nerves were cut and resutured. For a long time such an area responded only to crude pressure and crude thermal and pain stimuli, without spatial and gradual discrimination. Pain sensations were diffuse, poorly localized, highly unpleasant and with increased threshold. Fine tactile and temperature sensations, with sharp, point-like localization, and spatial and gradual discrimination did not reappear until several months after the nerve suture. On this basis the theory was advanced that there are two independent sensory fiber systems in the skin, the protopathic and the epicritic. The protopathic is the older and the less differentiated system. Acting alone it mediates explosive responses of an "all or nothing" type. The epicritic is the younger and more differentiated system. It serves to inhibit the abnormal intensity and faulty localization of the protopathic sensitivity.

Insufficient evidence is available for this theory and, in addition, factual discrepancies were found when Head's experiments were repeated (72, 63, 42). The strongest criticism was delivered by Lanier, Carney and Wilson (42), who repeated Head's experiments exactly and found facts which they felt differed in practically every point from Head's observations. Nonetheless, it is a conspicuous fact, and proves the inspirational value of Head's theory, that reference is still very

frequently made and consideration given to it in spite of the necessary reservations (2, 67), probably because, regardless of any theory, the existence of a protopathic sensitivity and its occurrence in a pure form is a clinical fact, familiar to every one who has to deal with sensory disturbances in lesions of the peripheral and central nervous systems. Even Lanier et al. under the heading of "Qualitative changes in sensitivity" describe "protopathic pain" in contrast with "normal pain" as an extremely uncomfortable and itching pain radiating over a wide area and tending to induce scratching. They also describe protopathic touch sensation. Finally they describe the hyperalgesic state in which incidental contact is felt as unpleasantly as in a bad sunburn. This state is very important in the understanding of the itch sensation. Of course, the term protopathic is used by these authors merely as descriptive and does not imply a special nervous mechanism. Their description, however, of the qualitative changes during nerve regeneration conforms to that of Head.

The main difference from Head's observations concerned the time relations in the return of protopathic and epicritic sensitivity. These time relations were such that in the interpretations of the authors, the theory of two independent fiber sets and the theory of suppression of protopathic sensations by epicritic sensitivity became untenable. In another part of this review, however, more recent experimental and clinical results will be reported which support both theories.

Itching and protopathic pain. The psychological analysis of the sensations, itching and protopathic pain, strongly suggested their close relation. Both protopathic pain and itching are characterized by poor localization, irradiation, poor discrimination of differential intensities, persistence of the sensation after the cessation of stimulation and by pronounced discomfort. On the contrary the epicritic pain sensation as elicited by sharp and short pin pricks is point-like, sharply localized and of short duration and has no similarity to the itch sensation.

Experimentally, when skin areas were tested whose epicritic sensory functions were lost in consequence of peripheral nerve lesions, distinction between itching and pain could not be made by the patient (57). With increasing intensity of the stimulus, itching went over into protopathic pain without any qualitative change of the character of sensation. Such a transition does not occur under normal conditions because the more intensive protopathic sensation is intermingled with, and more or less suppressed by, epicritic sensations. A weak pain which is

purely protopathic occurs more often in every day life and is identical with itching. In this conception scratching was interpreted as an expedient to suppress the very unpleasant protopathic impulses by the more tolerable epicritic pain.

Double pain sensation. In response to a single, short, pointlike, painful stimulus such as a pin prick a succession of two pain sensations can be observed. The two sensations are separated from each other by a sensationless time interval. The first pain sensation is short, pointlike, and can be sharply localized. The second is radiating, has an itching or burning character and is more unpleasant than the first (3, 49). In 1933 Zotterman (78) found that during the progressive loss of sensory functions of the skin induced by circulatory arrest of the limbs, there was a phase in which the reaction time of the pain sensation to a needle prick was increased to values of the same order as those found by Thunberg (70) for the second pain sensation. In other words, in local asphyxia a phase of sensory dissociation could be found in which the first pain was lost but not the second pain. The same type of sensory dissociation was studied in detail by Lewis and Pochin (49, 50), and they also found that under ischemia, loss of the first pain occurs much earlier than that of the second. Local anesthetics paralyze sensory functions in an order reverse to that of ischemia and hence, in cocaine anesthesia second pain is lost earlier than first pain. In circulatory arrest sensory qualities are lost in the following order: touch . . . cold and warmth . . . first pain . . . second pain. In local anesthesia second pain is lost first, and then first pain . . . cold and warmth . . . touch. These results could be related to findings of Clark, Hughes and Gasser (10) on the conduction rate of pain-mediating fibers. They found a close parallel in time relations between the successive disappearance of sensations in man during asphyxial nerve block and the successive disappearance of the electrical potential waves in electroneurograms of peripheral nerves in animals. It has been shown that the fibers of mixed peripheral nerves conduct afferent impulses at different conduction rates which are related to the size of the fibers. The A and B potential waves in the electroneurogram of peripheral nerves represent impulses of relatively large, fast-conducting fibers, whereas the late C waves are due to impulses in small fibers with a slow conduction rate. In human beings when, during asphyxial nerve block, sensations of pain and warmth alone can be evoked distal to such a block, in animals only C fibers remain active and their stimulation cause reflexes due to pain (10). It is true that there is a certain

overlapping of these pain responses towards the B waves, and it has been assumed that pain may be conveyed by both B and C fibers (28).

A much closer and clearer accord can be established, however, if C fibers are related not to pain generally but to second pain only because in ischemia C potential waves disappear at the same time that second pain disappears. Fibers of fast conduction rate and fibers conducting the first-pain impulses are more susceptible to local asphyxia. Fibers of slow conduction rate and fibers conducting second-pain impulses are more susceptible to cocaine (49). These results suggest that double pain sensations are due to impulses in two different sets of nerve fibers. The point-like, sharply localized first-pain sensation is conducted by fibers of fast conduction rate (B fibers); the second-pain sensation which has a burning, itching, radiating character follows later, because it is mediated by the small C fibers of slow conduction rate.

Definite evidence of two separate pain fiber sets was presented by Lewis and Pochin (49) when they showed that the time intervals between first and second pain progressively diminish with shortening of the pathway from the spot of stimulation to the central nervous system. Because the peripheral pathway is longest from the toes and fingers respectively, the time interval is longest if the pain stimulus is applied at these points. When one approaches the hip and shoulder, the peripheral pathway becomes shortened and the time interval diminishes steadily, until the difference in conductivity rate of the two pain conducting sets is not large enough to make two distinct sensations, and the first and second pain become entirely fused.

Pochin (54) also presented evidence that the delay of pain perception in tabes is due not to an abnormal slowing of impulses but to a defect in that group of pain fibers which conduct rapidly, and reveals the effects of a second group of fibers which conduct slowly (cf. 44).

Itching and tickling. It was mentioned on page 357 that earlier investigators were inclined to attribute "superficial tickling" to impulses arising in tactile receptors. The existence of specific tickle receptors has also been assumed (4). However, more recent clinical experiments showed that tickling sensation is intimately connected with the pain-mediating fiber system.

Pritchard (55), first stating that superficial tickle elicited by drawing a hair across the skin, is always highly unpleasant and leads to the motor response of rubbing or scratching, practically identified tickling with itching. In patients suffering from itching skin lesions he found

that itching attacks started with a tickling sensation which went over into itching without critical qualitative change of the sensation. Skin areas surrounding itching spots did not show hyperesthesia to touch but did show hypersensitivity to tickling. The threshold pressure necessary for tickling was not lowered, but, on equal stimulation, tickling was elicited more easily and more intensely around itching spots than in normal skin.

In patients suffering from peripheral neuritis, hypoesthesia to touch was combined with exaggerated responses to pain and tickle stimuli. Even when hypoalgesia was present, with its raised pain and tickle threshold, once the threshold was reached, the pain and tickle were unpleasant, almost to the point of being intolerable.

This hypoalgesia, in which tickle, itching (57) and pain responses are exaggerated, fits Head's description of protopathic sensitivity, with its raised thresholds and explosive sensory perceptions. On this basis tickle has to be regarded as a part of protopathic sensitivity.

Further close relations of tickling to pain have been revealed by Pritchard in his examinations of sensory disturbances in patients with lesions of the central nervous system. In all lesions of the spinal cord, thalamus and brain, pain and tickling were altered in parallel whether they were exaggerated or normal, weakened or totally absent. Thus, clinically, hyperalgesia may be detected by exaggerated tickling sensitivity, and absence of this sensitivity means impairment of pain conduction. Identical results were obtained by Foerster (21) in his neurosurgical experiments. Section of the anterolateral tract of the spinal cord abolishes tickling and itching. Such an operation at the corresponding level stops the most severe pruritus ani or pruritus vulvae immediately. In the corresponding area the patient feels normally and differentiates dull and sharp sensations but even the sharpest one does not cause any unpleasantness, and thus it cannot be called pain.

On the other hand, section of the posterior tracts in the acute experiment produces "hyperpathia," exaggerated tickle, itching and protopathic pain responses. "Posterior and anterolateral tracts serve both touch and pain, but the latter mediates only a primitive, nondifferentiated touch and pain sensation. The accessory impulses by which the differentiation of kind and origin of the tactile or pain stimulus is recognized, is due to impulses in the posterior tract" (21). These impulses exert a suppressing influence on the impulses of the anterolateral tract. The suppression occurs centrally in the thalamus and the cortex.

Recently on the basis of clinical experiments it was stated again that itching, if it is evoked by mechanical stimuli, always has a tickling component (37). The quoted results do not exclude the participation of tactile receptors in tickling impulses. Pritchard (55) considers the possibility that pressure receptors may initiate impulses which provide excitation of the pain pathway in the cord, if the frequency of repeated stimulation is sufficiently high. However, he points out that this supposition is in contradiction to Adrian's statement (2) that even the most frequent touch stimuli do not cause pain. It seems therefore more probable that the "tactile component" of the tickling impulse is set up in those pressure receptors which mediate primitive, nondifferentiated touch sensation, which have a low rate of adaptation, and which tend to summation and to produce after sensations. It will be shown in the next paragraph that the primary (protopathic) touch sensation of tickle has its counterpart in the electroneurogram of the impulse.

Electrophysiological analysis of itching (79, 80). All conclusions quoted in the foregoing paragraphs were more or less based on introspective observations of patients and normal test persons and might have been subject to error. However, application of the more recent methods of electrical registration of afferent nerve impulses as developed by the work of Adrian (2), Erlanger and Gasser (18), Bishop (9) and others, led to the same conclusions as did the clinical work, namely, that tickle, itch and protopathic pain are functions of the same set of nerve fibers. Such evidence was produced in animal experiments by analysis of the electroneurograms of impulses caused by different cutaneous stimuli which in their turn were analyzed for sensations they might cause in man.

In the experiments of Zotterman (79, 80), the impulses after the delivering of touch stimuli, such as moving of the hairs or painless deformation of the skin, showed in their electroneurograms large potential waves (A and B) which were due to impulses in fibers of more than 10 μ in diameter and of a conductivity rate of 30 to 60 meter/sec.

If pure noxious stimuli were applied without deformation of the skin, such as radiant heat or chemicals, the recorded amplitudes of the axon potentials were very low. They corresponded with the C waves of Erlanger and Gasser (18), i.e., with impulses of very small nonmyelinated fibers with diameters below 5.5 μ and a conduction rate of 0.7 to 1.3 meter/sec.

If touch or deformation was combined with pain stimuli, as in the case of pinching or pricking, there was a sudden response of large fibers

to the deformation and then a response of the very small fibers, which continued to respond in after-discharges with decreasing frequency.

Beyond that, any stimulus which elicited pricking pain was followed by "δ_1" potential waves representing impulses of fibers of less than 10 μ in diameter and of a conduction velocity of 20 to 30 meter/sec. These fibers are the largest in the spinothalamic tract and probably are myelinated. One found waves not only in responses to pin pricks but in responses to firm strokes as well. Thus, it seemed that the sharp "first pain" was due to impulses of specific fibers which always produced the same sensation independent of the kind of stimulus. On the contrary, the delayed "second pain," characterized by radiation, itching or burning quality and persistence after the stimulus had ceased, was due to slower impulses of non-myelinated fibers and appeared after any kind of burning stimulus and as an after-effect of needle pricks.

In tickle provoked by a light stroke with a piece of cotton, "δ_2" potential waves were recorded representing impulses of fibers of 5.5 to 7 μ in diameter and of a conduction rate of 8 to 17 meter/sec. Weak after-discharges in the C fibers corresponded with the itching after-sensation caused by such a stimulus.

The after-sensations following light strokes, firm strokes and burning stimuli on the skin could not be qualitatively differentiated. Itching and burning sensations fused as the strength of the stimulus was increased. In the electroneurogram all these impulses were represented by C potential waves. Their frequency increased with increasing intensity of the stimulus but their quality did not change. This means that *an increase in the stimulation of C fibers does not alter the character of the sensation when tickling progresses to itching and then to burning. The after-sensations of tickling and burning pain are identical with itching and are mediated by impulses of C fibers.*

Zotterman's work confirmed the law of specific energies distinguishing the potential waves belonging to fibers which mediate touch, on the one hand, and different kinds of pain, on the other. In 1935 Adrian (2) still considered the hypothesis of Goldscheider (29, 31) that both pain and touch sensations might be evoked by impulses in the same fibers, the difference in reaction depending only on the intensity of stimulation. Although it was clear at that time that the nervous apparatus responding to light touch did not mediate pain sensations and although evidence was presented that C fibers carried protopathic pain impulses, it was not clear that they conducted pain only. Thus the existence of specific fibers seemed to be doubtful.

In Zotterman's analysis the C fibers appear as carriers of uniform afferent impulses always causing the same quality of sensation independent of the kind of stimulation. Light and firm strokes as well as noxious pain stimuli have qualitatively the same late effect. Tickling, itching and protopathic pain are felt according to the frequency of impulses in the same fibers.

An objective basis for the poor localization of second pain and itching was found by Zotterman in polyphasic configurations of the corresponding axon potentials indicating the frequent branching of the axon.

Finally, the electroneurograms of Zotterman demonstrated the suppressive action of the fast epicritic impulses, travelling up the posterior columns, on the protopathic impulses which travel slowly in the anterolateral tract and arrive later in the center. This finding must be regarded as strong supporting evidence for the conception of Head.

Hyperalgesia (44, 45) *and itchy skin* (7). In 1916 Goldscheider (30) found that after crushing a circumscribed spot of the skin, a widespread area of surrounding hyperalgesia developed. In this area itching often was felt very unpleasantly, particularly after slight touching or stroking, and, as an after-sensation, to cold stimuli in this area.

The mechanism of this phenomenon was thoroughly investigated and clarified by Lewis in 1936 (44, 45, 46). He found that this hyperalgesia might be provoked: 1, by any painful injury of the skin; 2, by stimulation of a cutaneous nerve trunk, and 3, by stimulation of a small branch of a cutaneous nerve. In all three instances the hyperalgesia spread within or actually filled the cutaneous nerve territory in which the stimulus fell. The hyperalgesic area was more or less oval according to the shape and size of this territory. Its long axis was parallel to the nerve trunk and might be as long as 5 to 20 cm. The hyperalgesia was fully developed in 10 to 20 minutes and might last many hours. Needle pricks in this area caused unusually intense, diffuse and long lasting pain, whereas touch stimuli elicited soreness. The response to warmth was slightly increased.

This hyperalgesic state was not caused primarily by the effect of painful stimuli on the central nervous system. In other words, it was not referred from the brain or spinal cord. If block anesthesia was made previous to the crush so that the crush was unfelt, the usual area of hyperalgesia developed around the injury as soon as the nerve recovered from the block. The observation that the hyperalgesia closely filled the territory of the corresponding cutaneous nerve irrespective of the place and type of stimulation strongly suggested that the hyperalgesic state was established by a local nervous mechanism.

This was definitely proved by experiments with intradermal injections of local anesthetics. If a tiny crush was made in a small procaine wheal, hyperalgesia did not develop around the crush as long as the anesthesia persisted but developed in the usual way as soon as the skin recovered from anesthesia. Development and involution were simply postponed. Thus, hyperalgesia was not due to the spread of a pain producing substance into the neighboring skin for the diffusion of such a substance would not be hampered by block of the small nerve fibers which were hit by the local anesthesia. However, a persistent change was produced by the injury. The capacity to provoke hyperalgesia resided in and was maintained by the crushed skin; the effect of injury (chemical products?) acted through local nervous channels as soon as these became unblocked.

The action, as described by Lewis, occurred through local nervous channels and, being independent of the central nervous system, was explicable only on the basis of what has been called an axon reflex, the impulse traveling up one branch of an axon and returning from the point of ramification through side branches. Since the hyperalgesia originating in a small area might spread in all directions to include a much larger territory, it had to be assumed that a system of nerve fibers connected the small territory to almost every part of the larger hyperalgesic territory within the area of a given cutaneous nerve trunk. This system might be a network or a complex of overlapping arborizations of axons. The latter type was suggested on the basis of experiments with small novocaine barriers within the area of spreading. If the injury was made eccentrically within an intradermal novocaine wheal or outside but close to it, hyperalgesia developed to one side but failed to pass beyond the barrier until the effect of local anesthesia had worn off. The spread was blocked as soon as the way of the impulse from final arborizations to the parent axon was blocked. It did not creep around the barrier, as it would do if the impulse spread through a continuous network. Such a creeping, however, was observed when the injury was made far enough from the barrier so that the impulse might reach the point of ramification in the parent axon; then the impulse might return in every direction through side branches of the widely ramified axon. This type of impulse in widespread peripheral arborizations of a nerve trunk is identical with the type which must be assumed in order to explain the widespread pilomotor and sweat response to faradic stimulation and to intradermal injec-

tions of acetylcholine and other drugs with nicotine-like action (48, 8, 75, 13, 60).

Lewis (44) has shown that the hyperalgesic state as described above is not a function of sympathetic fibers. It occurs unchanged in areas deprived of sympathetic innervation. Lewis also dismisses the rôle of sensory nerves in the development of the hyperalgesic state for two reasons: first, he regards pain as an accurately located sensation and states that such a sensation cannot be elicited in a system of branching axons. Secondly, he finds that in sensory dissociation by circulatory arrest and by local anesthetics the disappearance of pain perception and of the hyperalgesic state are separate. "Cutaneous pain nerves merely register, through sensations called hyperalgesia, a state of the skin for which they themselves are in no way responsible." Lewis assumes the existence in the skin of a hitherto unknown nervous system and discusses the possibility of its identity with nerve plexuses involved in antidromic vasodilatation and in the state of erythralgia (43). Particularly emphasized is the similar behavior of the "flare" and the hyperalgesia, both spreading around local skin injuries in a similar distribution. The nervous system in question is associated with local defense against injury by protective hyperalgesia and by protective hyperemia and is therefore appropriately called the "nocifensor" system.

To exclude the participation of pain fibers in the mechanism which leads to hyperalgesia for the reason that pain sensations are sharply localized, whereas the hyperalgesia is based on a special state of widely ramifying axons, seems to be correct concerning epicritic pain but can hardly be accepted in regard to the protopathic quality. On the contrary, according to the description the hyperalgesia around the point of painful stimulation is identical with a state which might be called "protopathic hyperalgesia" or "hyperpathia" (21) and which is well known and described in lesions of the peripheral and central nervous system as well as in itching skin lesions, particularly in gross lesions of the epidermis ("eczematiform" reactions). Lewis' second reason for ruling out sensory nerve fibers is not easy to eliminate. He finds that, whereas the second pain sensation is the first sensory perception to be lost in cocaine anesthesia and the last one to be lost in asphyxia, the capacity for developing a hyperalgesic state shows an almost opposite behavior, namely, its very early loss in asphyxia and its late loss in cocaine anesthesia. What is more, the experiments of Lewis prove

definitely that asphyxia does not interfere in any way with the mechanism that produces the hyperalgesia but acts directly on the "nocifensor" nerves themselves. It must be admitted that this peculiar behavior can hardly be explained otherwise than by the existence of a system independent of pain perception.

Even if in the future a simpler explanation were found for the different behavior of protopathic pain and hyperalgesia in circulatory arrest and in local anesthesia, and even if hyperalgesia were recognized as a special hyperactive state of the slow conducting C fibers, as believed by the reviewer, it still would remain an important discovery that the nervous apparatus in question forms a branching and rebranching system of axons within the territory of a cutaneous nerve. By this statement, as previously mentioned, a remarkable similarity with the sympathetic fibers of the skin has been established and it may be pointed out that this similarity seems to concern *two phylogenetically older and less differentiated systems of small and unmyelinated fibers, which mediate diffuse effects, in contrast to the newer and highly differentiated epicritic sensory system.* The capacity of sharp localization in the latter is evidently incompatible with ramifications of the axons. The type of diffuse and exaggerated sensory responses as they are frequently found in pathologic conditions of the nervous system, skin and mucous membranes is more clearly understood by an acknowledgment of the findings of Lewis.

The close relation of these findings to the problems of itching became evident by Bickford's observations on "itchy skin." Bickford (7) found that after applying a stimulus which causes itching, two different phenomena occurred: 1, there was a local itching confined to the point of stimulation which persisted, after the stimulation had ceased, without the intervention of further stimuli and was therefore called "spontaneous itching"; 2, around the point of stimulation a widespread area could be mapped out which did not itch spontaneously but responded with itching to light friction. This area was called "itchy skin." In complete accord with the hyperalgesic area of Lewis the area of itchy skin was oval shaped with its long axis parallel to the corresponding cutaneous nerve trunk and it filled closely the territory of the nerve. Evidence has been presented that it spreads through axon ramifications as the hyperalgesia does.

The close relation between hyperalgesia and itchy skin is indicated by their alternating appearance in the same individual after more or less painful stimulation. There are also individual differences in the

response to identical stimuli, some persons signaling hyperalgesia, others, itchy skin. Furthermore the hyperalgesic area might be surrounded by a narrow zone of itchy skin (44, 7).

"Spontaneous itch" and "itchy skin" could be separated in sensory dissociation caused by local asphyxia and by local anesthetics exactly in the same way as pain and hyperalgesia.

"Itchy skin" playing a prominent rôle in skin diseases has been known and described long ago under the heading of "increased itching sensitivity" (58, 59). It has been stated that "itching skin lesions," such as urticarial wheals, prurigo nodules, eczematiform vesicles and lichenified lesions, itch "spontaneously" only during their development while irritating substances are being formed locally by the morbid process. These irritating substances are responsible causally for two independent phenomena: 1, the anatomic (inflammatory) lesion itself, provoked by cell injury, and 2, the itching, provoked by stimulation of the nerve endings. This itching is of short duration, usually lasting only a few minutes. However, after this itching has subsided an increased itching sensitivity persists for many hours or even days, in the area of the lesions, regardless of whether the anatomic lesion has subsided, as in urticaria, or has not, as in the case of eczema.

Even if the increased sensitivity is not very pronounced, as in urticaria, itching may always be elicited by rubbing, hours or days after the urticaria has subsided. This phenomenon is a part of what has been called somewhat poetically "mnemodermia" or cutaneous memory (35, 68). There is no itching skin disease in which it cannot be found.

If the persisting excitability is high, the threshold for adequate stimuli is considerably lowered and the responses are exaggerated. In addition, inadequate stimuli, such as light touch, light strokes, pressure, release of pressure and temperature stimuli, elicit violent itch sensations. In such spots, one might say, even the slightest breath of air may evoke itching, and, under the conditions of every-day life, it becomes practically impossible to avoid all the trivial stimuli causing the itch sensation. Although in reality this condition is a state of "itchy skin," or itching hyperexcitability, it is mistaken for a state of "spontaneous itch" because the slight and inadequate, but very effective, external stimuli escape notice (58, 59).

The responses of itchy skin areas to different stimuli were as follows: 1, needle prick revealed definite hyperalgesia, intensification, prolongation and radiation of the pain sensation, and itching after-sensation; 2, itch powder caused greatly intensified, radiating and prolonged itching;

3, light stroke and pressure might evoke characteristic itch sensation (58); 4, cold stimuli might cause itching or stinging pain (30).

Again, these responses show the features of a skin area with merely protopathic sensitivity, as it is observed in lesions of the peripheral and central nervous system. This condition can hardly be explained except by a decrease of epicritic sensitivity in consequence of injury which may act 1, on the nerve endings within the skin; 2, on the peripheral nerve trunk, and 3, on the central pathways, always with the result of an exaggerated display of protopathic sensitivity. If "hypoalgesia" is recorded in these cases, it means raised threshold of epicritic pain. The threshold of protopathic sensitivity may also be high but once reached, the response is explosive.

The distinction between "spontaneous itch" and "itchy skin" by Bickford (7) has its counterpart in the quality of the respective sensations. That provoked by external stimuli in itchy skin represents pure itching, or, if the stimulus is intensified, the sensation is a diffuse burning, itching pain. In spontaneous itch an admixture of point-like pricking sensation is felt primarily, most conspicuously in experiments with itch powder (57), but is observed also during the eruption of skin lesions and in itching elicited by histamine. This epicritic component of spontaneous itch gradually fades away so that only pure itching remains and, after this is gone, itchy skin results. Thus, spontaneous itch can be defined as a mixture of epicritic and protopathic pain impulses of slight intensity or, according to Zotterman (79), as a mixture of impulses in δ and C fibers, whereas itchy skin represents a hyperexcitability of protopathic C fibers only. This view cannot be brought into accord with Bickford's sensory dissociation experiments in asphyxia block, the results of which are practically the same as in Lewis' hyperalgesia experiments, showing discordance in the disappearance of spontaneous itch and of itchy skin. However, the view is in accord with Bickford's observations in patients with lesions of the central nervous system causing sensory dissociation, experiments which, the reviewer believes, offer more natural and simpler conditions than asphyxia. Altogether in these experiments tickle, itchy skin, spontaneous itching and pain tend to disappear as a group after chordotomy and in destructive diseases of the spinal cord, if the anterolateral tract is involved.

Itching and vasomotor action. From the initial experimental research on itching up to recent times, the theory has often been advanced that impulses in vasomotor fibers participate in evoking itching sensation and that vasomotor reactions are responsible for its peculiar character

(59). This theory has held sway because itching is often accompanied by local vasomotor reactions, either in the form of vasoconstriction with pilomotor reaction or vasodilatation with or without urticarial whealing. Moreover, itching dermatoses are frequently associated with general disturbances in the autonomic nervous system, and in such instances itching responds to a certain degree to treatment with epinephrine or atropine. The analysis of the available data led the reviewer (59) to the conclusion that vasomotor phenomena are either coördinated with itching deriving from a common cause or that they are a reaction to the sensory impulse analogous to vasomotor reflex phenomena which follow pain sensations (74). Recently this view was challenged (10, 36), mainly on the basis that itching cannot be elicited in a skin area where the minute vessels have been contracted by a local injection of epinephrine. It has been said that in order to evoke itching a neuro-vascular change, either dilatation or constriction, is necessary, and this is inhibited by the spasm caused by epinephrine. However, it was not considered that the local ischemia following the epinephrine injection might be the reason for the loss of itching sensitivity. The reviewer interprets the results of the epinephrine experiments as evidence that sufficient blood circulation is required to bring about itching sensation. However, he does not see any proof for the need of any special vasomotor change.

After it has been shown that itching arises as readily in skin areas deprived of sympathetic innervation as in normal skin, the rôle of sympathetic fibers in producing itching sensation has been definitely ruled out. Still, the interference of antidromic vasodilatation is to be considered because it often occurs with itching as a coupled response. However, evidence for influencing the quality of itching sensation by antidromic vasodilatation has not been brought forth up to the present time.

Chemical mediation of itching. The study by Lewis and his collaborators of the triple response of the skin to chemical, mechanical and other physical injuries (43) has led to the conclusion that this response is due to the release of histamine or a histamine-like substance ("H-substance"). This substance, liberated from the injured cells of the epidermis and entering the intercellular spaces has three independent actions: 1, it dilates the minute vessels of the skin (local redness); 2, increases the permeability of these vessels (local wheal formation); 3, sets up an arteriolar dilatation by means of an axon reflex mechanism in posterior root fibers (flare around the wheal).

As the triple response is always accompanied by itching the question has arisen whether this sensation, too, may not be provoked by the release of H-substance. The idea was that possibly any physical or chemical stimulus which provokes both the triple response and itching, does not act directly on cells and nerve endings but through the mediation of the released H-substance. The supporting evidence for the theory that itching is effected through chemical mediation was presented by Lewis, Grant and Marvin (47) who showed that if the circulation is arrested in one arm and then both arms are uniformly stimulated the itching is intensified and greatly prolonged on the arm with arrested circulation. The period of itching may be prolonged by the whole time duration of circulatory arrest. The interpretation of this observation was that "the released substance causing itching is held in situ and continues to act on nerve endings during circulatory arrest" (43).

The conception of a uniform chemical mediation of the triple response and of itching had a fertile influence on the interpretation of pathologic processes in the skin, particularly when the observations were extended to the responses in anaphylactic and allergic skin reactions (43). These reactions and the responses to histamine showed an identical behavior under various conditions and it has thus been assumed that the anaphylactic or allergic antigen-antibody reaction in the skin leads primarily to the liberation of H-substance in the same way as injury does. This conception has been of great importance in respect to itching because a great number of itching skin lesions are based on hypersensitivity reactions (59). The two prototypes of such lesions are: 1, the urticaria, in which the site of the hypothetical sessile antibodies is the cutaneous vessel wall (urticaria is a "local anaphylactic shock," 15), and 2, the eczematiform vesicles, in which the site of hypersensitivity is supposed to be in the epidermal cells. A further fact in favor of the histamine theory of itching in anaphylactic reactions is that pruritus may be the first or even the only symptom of the systemic anaphylactic shock in animal experiments as well as in human beings (39). This itching might be related to increased histamine content in the circulating blood during the anaphylactic shock (19).

It seems compatible with all known facts to assume that in itching cutaneous reactions based on hypersensitivity, itching is mediated by the liberation of histamine or a similar substance. According to this view anaphylactic or allergic skin reactions and itching are coördinated phenomena, both caused by the same chemical agent. However it must be emphasized that all evidence for this view, though impressive, has been indirect. Attempts to isolate histamine or a similar substance

from these lesions have not been successful. For this reason the term "H-substance" was chosen.

Direct evidence for liberation of histamine in excised pieces of skin when they are subjected to painful electrical stimulation has been presented recently (56). The experiments strongly support the theory that liberated histamine may be a chemical mediator for cutaneous pain.

In this whole problem one thing is certain, namely, that neither itching nor cutaneous pain is mediated in every instance by histamine or a similar substance. This conclusion can be drawn from the fact that histamine in as high a dilution as 1:600,000 to 1:1,000,000 still causes whealing (43); this dilution, however, does not cause either pain or itching in a higher degree than physiological saline. It appears that histamine can be responsible for cutaneous pain or itching only in those instances in which they are accompanied by whealing or other inflammatory changes according to the site of liberation.

Kenedy (37) has discussed the most likely possibility, that itching may be mediated by histamine in certain cases but not in others. Many conditions are known to cause violent itching without any gross or microscopic changes of the skin; such as the pruritus in pregnancy, diabetes, diseases of the liver and of the hematopoietic system. Whether in these cases the metabolic products of the pathologic process act directly on the nerve endings or by intervention of a chemical mediator, is not known but it can be stated that histamine cannot be the mediator because nothing is seen on the skin. In concentrations which evoke itching, histamine causes visible changes. "Pruritus sine materia" (pathologic itching without visible changes) is not mediated by histamine.

The scratch reflex. The motor response to itching is a spinal reflex and can be elicited in the spinal dog from a saddle-shaped area of the back by mechanical or electrical stimulation (64, 14), and in the cat by mechanical stimulation of the external ear (11). The scratch movements are carried out with the homolateral hind limb. The rhythm of the movement is based on refractory periods developed in spinal centers and is independent of the frequency of the stimulus (64). The reflex is disynaptic and is brought about through the anterolateral and anterior tracts of the spinal cord (64). After bilateral ablation of the frontal cortex the scratch reflex becomes considerably enhanced (11). Thus the existence of an inhibitory mechanism of higher centers on the scratch reflex has been established.

Itching and scratching can be provoked in cats also by partial sensory

denervation producing a state of "itchy skin" or protopathic hypersensitivity. In this condition scratching is so violent that large pieces of skin are torn off and deep excoriations are made which heal with scars (5).

In human beings the different forms of the motor response to itching, such as scratching with the nails, rubbing or kneading the skin, are controlled to a certain degree by consciousness and will power similarly to the defense reflex to pain stimuli. Itching was interpreted as a "foreign body sensation," and scratching, teleologically, as a suitable defense reflex for removal of noxious foreign bodies (53).

There are two instances in which pathologic itching may be stopped by scratching: first, when itching is actually caused by foreign bodies which can be removed from the skin by scratching, and second, when, by scratching, the epidermis, in its entire thickness (and with it the epidermal nerve fiber nets) is torn off. This happens in cutaneous diseases either by tearing off elevated lesions (e.g., prurigo papules) or by tearing off the flat skin surface with "digging" movements of the nails (e.g., neurotic excoriations). In these cases itching ceases immediately in loco.

In all other instances scratching is unsuited for stopping itching. As long as one scratches, there is relief because the more tolerable epicritic pain suppresses the intolerable itching. However, when the scratching is discontinued the itching starts again as an after-sensation following scratching or rubbing stimuli because these stimuli send impulses not only through epicritic but also through protopathic fibers. In pathological cases of "itchy skin" in which the protopathic sensitivity is over-accentuated, this situation leads to a tormenting vicious circle, consisting of increasingly violent scratching and increasingly intensive itching. Such scratch paroxysms may last many hours and may cease only because of total somatic and psychic exhaustion of the patient.[1] In chronic cases a further disturbing factor must be taken in account, namely, a special proliferative reaction of the epidermis to chronic mechanical stimulation called "lichenification," which exaggerates the hyperexcitability of the protopathic system. Whether this state is connected with new formation of nerve plexuses in the epidermis is not known but seems to be possible.

Itching of central origin. Centrally induced itching was observed

[1] Discussion of the alleged relation of scratching to sexual instincts (scratching a "cutaneous onanism" (35), "substitutive for sexual orgasm" (65)) are beyond the scope of this review. For details see (62, 66).

after intracisternal injection in the cat of 0.2 to 0.5 mgm. of morphine (52, 41, 76). Violent scratching, mainly of the ears but sometimes of the nose and other parts of the head and neck, by one of the hind limbs started a few minutes after the injection and lasted for from 1 to 1½ hours. After the attack subsided, a new attack of scratching could be provoked by stroking the ear. After local anesthesia of the ear or after section of its sensory nerves this peripheral inducement to scratching failed, but the effect of renewed intracisternal injection of morphine was not impaired. It was thus shown that the effect of morphine is directly on the center. Intralumbar, intravertebral and paravertebral injections of morphine were without effect.

Successive ablation of cortex, thalamus, corpus striatum and pallidum, midbrain, cerebellum and medulla to the level of the acoustic nucleus, did not influence the scratch response. If the medulla was sectioned at the level of the restiform bodies and the injection was made into the opened cerebrospinal canal rhythmic movements of the hind limb were still observed but the limb did not reach the head. After sectioning of the medulla at the level of the pyramid decussation real scratch movements were no longer seen, but some uncertain movements occurred. It was concluded that the main site of the scratch center ("Kratzwerk") is situated in the medulla below the acoustic nucleus.

In human beings itching evoked without peripheral impulses has been observed in diseases of the central nervous system, such as tabes, dementia paralytica, and more rarely in manic-depressive psychosis, idiocy and epilepsy.

The excitability of the central nervous system also influences the intensity of any kind of peripherally induced itching. If the itching is not very intense the attention can be diverted from it (77). On the other hand merely imagining the presence of biting insects may provoke itching and scratching in centrally hyperperceptive individuals.[2] Pharmacologically, caffeine intensifies the central perceptibility of itching (59), whereas bromides and the narcotics acting on the brain stem (phenobarbital and other barbiturates) have a beneficially depressing effect (17, 40).

It is an unexplained fact that morphine and the related derivatives of opium, the most effective drugs for central relief of pain, do not decrease the itching sensitivity but, on the contrary, usually increase it. Even in morphine-induced deep sleep scratch movements can be

[2] The relation of itching to functional skin diseases has been discussed in detail by Becker and Obermayer (6).

observed in patients suffering from itching skin diseases. Probably this itching is partly due to a peripherally stimulating effect of morphine, as morphine given intracutaneously elicits the triple response of Lewis with itching. Another possibility is that morphine may provide a state of cutaneous hypoalgesia in which pain stimuli tend to cause itching sensations (see p. 359). However a central stimulating effect on itching must also be taken in account on the basis of animal experiments quoted above.

As previously mentioned, local injection of epinephrine inhibits responses to itch stimuli. However, if itching has once been aroused in normal skin, the subsequent local administration of epinephrine does not inhibit further itching (10). This observation demonstrates the central summation of itching which may be compared with the central summation of pain (1).

Therapeutic considerations. If the causal factors in the development of an itching skin disease cannot be determined, the physician is compelled to treat itching as one of the predominant, troublesome and, because of the scratch reflex, intractable symptoms.

According to what has been said about "itchy skin" or itching hypersensitivity, there are, besides the necessity of decreasing central sensitivity, two principles governing such treatment: 1, reducing the visible lesions which are the basis of itching hypersensitiveness; 2, eliminating external stimuli as far as possible by use of suitable non-irritating clothing; by avoiding gross temperature changes; controlling sudden changes in pressure as they occur in undressing, in removing bandages, and in entering and leaving the bath. The understanding of itching hypersensitivity as a state in which inadequate stimuli elicit itching is of great importance in therapy (59).

SUMMARY

Itching represents a sensory quality which is identical with that of protopathic pain. Superficial tickling progresses to itching and itching to diffuse burning pain without critical change of the quality of sensation when the intensity of the stimulus is gradually increased. They have a longer latent period than other cutaneous sensations because they are mediated by the slowly conducting C fibers of Gasser and Erlanger. Whether tickling, itching or protopathic pain sensation is felt, depends on the frequency of impulses in these fibers. The sensations persist after the cessation of stimulation, the fibers being inclined to after-discharges and their impulses being capable of central summa-

tion. They often appear as after-sensations of other sensory perceptions. The clearest example of such combined impulses in different fiber sets is the double pain sensation following needle pricks.

Itching is experienced in a rather pure form, and becomes accentuated or explosive if the epicritic sensitivity is depressed following injuries to the skin, to the peripheral nerve trunk, or to the central nervous system. Itching caused by pathologic processes is always based on such a condition. If the over-accentuation of protopathic sensitivity is pronounced, the slightest external stimuli, even if they are inadequate (e.g., touch and temperature stimuli) evoke itching.

Polyphasic configurations in the electroneurogram of "second pain" impulses indicate the frequent branching of the corresponding axons. The ramifying axon system revealed by Lewis in experiments on hyperalgesia, although interpreted differently, also seems to be related to the perception of protopathic pain and itching. Radiation and poor localization of these sensations are explained by their originating from richly ramifying axons. According to this view there are three ramifying axon systems in the skin, the sympathetic, the antidromic vasodilator and the protopathic sensory system, all consisting of small unmyelinated fibers and all mediating diffuse effects by means of axon reflex impulses. Sympathetic and protopathic sensory systems often respond in coupled reactions to cutaneous stimuli but itching arises without any interference of the sympathetic fibers. Itching and antidromic vasodilatation are also frequently coupled with each other but there is no evidence that vasodilatation is a necessary factor in evoking itching.

Supporting evidence has been presented for the theory that itching following skin injury and itching in skin lesions based on hypersensitivity reactions might be mediated by liberation of H-substance. However, liberation of histamine or of a similar substance cannot be responsible for itching which occurs without visible changes in the skin.

The central inducement of itching, the scratch reflex and therapeutic considerations are briefly discussed.

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